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A role of mast cells in women health and disorders of the endometrium

Bianca De Leo MPharm MSc

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Declaration

The studies presented herein were unaided work of the author. The work described in the thesis has not been submitted to any other degree or professional qualification.

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Abstract

During the normal menstrual cycle, the human endometrium undergoes extensive tissue remodelling under the influence of ovarian-derived hormones. The endometrium has well defined stromal and epithelial compartments with the former containing both a well-developed vasculature as well as a diverse population of immune cells. Mast cells (MCs) are long-lived tissue resident immune cells characterised by the presence of granules containing proteases. Mast cells have been detected in the human uterus but little is known about their regulation or the impact of steroids on their differentiation status. Recently MCs have been implicated as key players in physiological and pathological pain pathways but little is known about their role in endometrial pathologies. Endometriosis is a chronic incurable condition characterized by the presence of endometrial tissue outside the uterine cavity: women with endometriosis can suffer from a debilitating range of symptoms including chronic pain. Whilst the aetiology of endometriosis is uncertain, close proximity between MCs and nerves has implicated them in aberrant activation of pain pathways.

The aims of the current project were: 1. To determine the spatial and temporal location of uterine MCs and to explore their phenotype including expression of steroid receptors. 2. To explore the activation status of MCs in women with endometriosis and/or pain, 3. To explore the use of cells and mice as models to investigate the phenotype of mast cells and their regulation by steroids.

Mast cell proteases tryptase and chymase were detected by RTPCR and immunohistochemistry in “full thickness” (uterine lumen to endometrial-myometrial junction) biopsies from women undergoing hysterectomy. In agreement with previous findings MCs were most abundant in the myometrium. Uterine MCs were predominantly of the classical MC subtypes: tryptase^{pos}/chymase^{neg} and tryptase^{pos}/chymase^{pos} but a rare third subtype was also identified as tryptase^{neg}/chymase^{pos}. Mast cell activation/degranulation was cycle stage dependent and for the first time their steroid receptor phenotype was identified as ERα^{neg}/ERβ^{pos}/GR^{pos}, suggesting potential regulation by the uterine steroid microenvironment.

Studies on tissue samples from women with endometriosis revealed MCs with an altered activation status in the pelvic peritoneal wall, compared to controls, which showed an intense diffuse immunoexpression of chymase suggestive of MC activation and release of this protease during normal physiology of the peritoneum. Surprisingly, analysis of peritoneal fluids from controls, women with pain but no endometriosis, and pain with endometriosis did not detect differences in numbers of MCs or concentrations of tryptase or chymase. Analysis of peritoneal biopsies also provided the first evidence for a striking increase in immunoexpression of PAR-2, a protease-activated receptor, in women suffering from chronic pelvic pain and/or endometriosis which may provide a mechanism by which mast cell derived factors may alter pain pathways.

Studies in a mouse model of endometriosis identified MCs within endometrial-like lesions and offer a platform for future studies. *In vitro* explorations using MCs derived from peripheral blood precursors and HMC-1, a cell line derived from a patient with MC leukaemia confirmed expression of ERβ but did not support previous studies claiming cells were $ER\alpha^{pos}$.

In summary, this study has provided novel insights into the phenotype of endometrial mast cells in the normal cycling endometrium and contrasted them with those in women with endometriosis and pelvic pain. This is the first study to identify MCs as $ER\beta^{pos}$. Further studies are required to determine whether inhibition of PAR-2 might offer a therapeutic target in women with chronic pelvic pain.

Layman Abstract

Mast cells (MCs) are cells of our immune system, which are considered to be the main players in common allergic reactions such as itch and pain but can also be involved in asthma. However, MCs are not just “allergy cells”, as they are versatile and present in almost every organ of the body supporting other aspects of our health. For example, MCs, together with other immune cells, are resident in the different layers of the human womb. They are thought to help the inner layer of the womb termed endometrium to shed, which is a key event in the menstrual cycle. Understanding how MCs behave during normal conditions can help us to comprehend their role in different diseases. Endometriosis is a condition recognised as a social problem because it affects one in ten young women and impacts their quality of life. Although, approximately 50% of women with endometriosis experience constant and debilitating pain that drastically affects their lives, an efficient treatment for endometriosis has yet to be found. Recent studies reported the presence of MCs in endometriosis lesions, which are small pieces of tissue that behaves like the endometrium outside the womb. Specifically, MCs were found in lesions growing on the thin surface of the abdomen (the peritoneum) that covers and keeps the internal organs together. These lesions are the real cause of the disease and its pain.

MCs consist of well-organised granules, which act as “storage boxes” for all the molecules they need in order to do their job. When MCs receive a stimulus, they release the granule content. MCs are the only immune cells that can produce, store and release two particular molecules: tryptase and chymase. It is thought that female hormones called oestrogens can stimulate MCs. Therefore, the aim of this project was to study whether MCs are equipped with tools to be able to respond to oestrogens. In particular, we examined whether MCs have oestrogen receptors, which act as the “lock” for the “key” oestrogens to activate MCs. Indeed, this research has revealed that MCs in the normal endometrium expressed only one of oestrogen receptors, the β form and not the α form, which others had previously reported. MCs have also been found in the endometrium from women with endometriosis, and they looked different: higher in numbers and containing both tryptase and chymase, a feature not present in those detected in a normal uterine tissue during this study. Moreover, this study

confirmed the presence of MCs in the endometriotic lesions, which were in an activated state with chymase located outside of the cell and in the surrounding tissue.

This project also studied the population of MCs and the levels of tryptase and chymase that were present in the peritoneal fluid of patients suffering from endometriosis. Peritoneal fluid is a small volume of liquid that surrounds the organs and help them to avoid friction and distress. Unexpectedly, lower number of MCs was detected in the fluid of endometriosis sufferers compared to unaffected women. Endometriosis patients also had a lower amount of tryptase and chymase in their fluid. Once tryptase is released from the cytoplasm of MCs, it can interact with a specific protein, called PAR-2. PAR-2 is a receptor, which is in turn triggered by tryptase. Tryptase functions like a "pair of scissors" and cuts a small string of amino acids from PAR-2 protein. The latter subsequently enables PAR-2 to action further. The presence of PAR-2 may reflect a mediator role in a chain reaction that follows MC activation and PAR-2 is reportedly involved in the perception of pain. Our results indicate extremely higher concentration of PAR-2 in the abdomen of women with endometriosis compared to healthy women.

Lastly, another aim of this project was to model possible MCs behaviours with cells grown of a dish, as well with animals, which mimic the endometriosis condition and symptoms. Taken together, our results provide an excellent stepping-stone for future studies on MC-targeted endometriosis therapeutics.

In summary, this study has carefully described MCs in the normal womb and showed for the first time that these cells are different in women suffering from endometriosis compared to healthy women, both in the womb and in peritoneum, suggesting that MCs may be involved in endometriosis-related and chronic pain. Further work will help inform on the role of MCs in endometriosis.

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List of abbreviations

HSD	Hydroxysteroid dehydrogenase
2D	Two dimensional
3D	Three dimensional
ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
APC	Allophycocyanin
ASRM	American society for Reproductive Medicine
bFGF	Basic fibroblast growth factor
BMMCs	bone marrow derived mast cells
BSA	bovine serum albumine
BV	brilliant violet
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CBMCs	Cord blood derived MCs
CCL	CC chemokine ligand
CD117	(also known as c-kit or SFCR) stem cell factor receptor
CD11b	Antigen-like family member B
CD133	Nectin-3
CD14	Cluster of differentiation 14
CD16	Cluster of differentiation 16, low affinity Fc receptor, surface marker for NKs, neutrophils, monocytes, macrophages.
CD163	Cluster of differentiation 163, marker monocyte/macrophage lineage
CD19	Cluster of differentiation 19, B-lymphocyte antigen
CD3	Cluster of differentiation 3, marker for T-lymphocytes
CD34	Hamatopoietic progenitor cell antigen
CD45	Leukocyte common antigen
CD56	(also known as NCAM), surface marker for NKs
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2 enzyme
CPP	Chronic pelvic pain
Ct	Cycle threshold
CTMCs	Connective mast cells
CXCL	Chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptors
CYC	Cyclophilin
CYP11a	Cytochrome P450 11a or cholesterol side-chain cleavage enzyme
CYP17	Cytochrome P450 17A1 or 17α-hydroxylase
CYP19	Cytochrome P450 19 or aromatase
DAB	3, 3'-diaminobenzidine
DAG	diacyl glycerol
Dapi	4,6'-diamidino-2-phenylindole
DBD-C	DNA-binding domain
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPN	Diarylpriopionitril
DTT	Dithiothreitol
E_2	17β-oestradiol
ECM	Extracellular matrix
ECP	Eosinophil specific cationic protein
ELISA	Enzyme-linked immunosorbent assay
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ERE	Oestrogen response elements
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FACS	fluorescence-activated cell sorting
FAM	6-carboxyfluorescein
FcεRIα	High affinity immunoglobulin epsilon receptor subunit alpha
FMO	Fluorescence minus one
FOXO3a	Forkhead box O3

FSC-A	Forward scatter area
FSC-H	Forward scatter height
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GnRH	Gonadotropin-releasing hormone
GPCR	G protein–coupled receptor
GRα - β	Glucocorticoid receptor alpha and beta
GRE	Glucocorticoid response elements
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen peroxide
HCl	Cloridric acid
HEECs	Endometrial endothelial cells
HEPES	4-(2-hydoxyethyl)-1- piperazineethanesulfonic acid
HESCs	Endometrial stromal cells
HIF-1 α	Hypoxia-inducible factor 1-alpha
HLADR	Human Leukocyte Antigen - antigen D Related
HMC-1	Human mast cell cell line
HMC-1.1/2	HMC cell line variants with one point mutation (V560G) or two point mutations (V560G and D816V)
HR1	Histamine receptor 1
HRP	Horseradish peroxidase conjugated
i.p.	Intra peritoneal injection
IBS	Irritable bowel syndrome
IC	Interstitial cystitis
ICI 182,780	Fulvestrant
IF	Immunofluorescence
Ig	Immunoglobulin
IHC	immunohistochemistry
IL	Interleukin
IFNα-β	Interferon alpha and beta
IP3	inositol-1, 4,5-triphosphate
LAD2	Leukocyte Adhesion Deficiency derived cell line
LapSter	Laparoscopic sterilization
LapSterRev	Laparoscopic sterilization reversal
LBD-E	Ligand binding domain
LCT_4	Leukotriene C4
LDS	Lithium dodecyl sulphate
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LIF	Leukemia inhibitory factor
LNAs	Locked nucleic acids
LNG-IUS	Levonorgestrel-releasing intrauterine system
LTB_4	Leukotriene B4
MC_C	Mast cell tryptase positive
mCPA	Carboxypeptidase A
MAPK	Mitogen-activated protein kinase
MCs	Mast cells
MC_T	Mast cell tryptase positive
MC_{TC}	Mast cell tryptase and chymase positive
mMCP	Mast cell protease
MMCs	Mucosal mast cells
MMPs	Matrix metalloproteinase
mRNA	Messenger RNA
n.a.	Not applicable
n.d.	Not detected
NaCl	Sodium cloride
NBF	Neutral buffered formalin
NGF	Nerve growth factor
NGS	Normal goat serum
NK	Not known
NK-1	Neurokinin 1, substance P receptor
NO	Nitric oxide

No RT	No reverse transcriptase control
NSAID	Non-steroidal anti-inflammatory drugs
OD	Optical density
P_4	Progesterone
PAF	Platelet activator factor
PAR-2	Protease-activated receptor 2
PBMCs	Peripheral blood derived MCs
pbNKs	Peripheral blood NKs
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PF	Peritoneal fluid
PFA	Paraformaldehyde
PFR	Pelvic floor repair
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
$PGF_2\alpha$	Prostaglandin F2α
PGs	Prostaglandins
PLGF	Placental growth factor
PMCs	Peritoneal mast cells
PPT	Propyl-pyrazol triol
PR-A, B	Progesterone receptor alpha and beta
PRE	Progesterone response elements
pro-MMPs	Pro matrix metalloproteinases
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
ROI	Region of interest
rSC	Relative standard curve
RT	Room temperature
s.c.	Subcutaneously
SCF	Stem cell factor
SEM	Scanning electron microscopy
SERMs	Specific oestrogen receptor modulators
SGRMs	Selective glucocorticoid receptor modulators
SP	Substance P
SPRMs	Selective progesterone receptor modulators
SSC	Side scatter
TAH	Total abdominal hysterectomy
TB	Toluidine blue
TBE	Tri-borate EDTA (ethylene diamine tetra-acetic)
TBS	Tris buffered saline
TGFβ	Transforming growth factor beta
TMB	3,3'-5,5'-tetramethylbenzidine
TNFα	Tumour necrosis factors alpha
trkA	Tropomyosin receptor kinase A
TRL	Toll-like receptor ligands
tRNA	Transfer RNA
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
TRPV4	Transient receptor potential cation channel subfamily V, member 4
TSA	Tyramide signal amplification
uNKs	Uterine natural killer cells
UPL	Universal probe library
UV	Ultra violet light
VagHyst	Vaginal hysterectomy
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
WB	Western blotting

Chapter 1 Literature review

1.1 The endometrium and the menstrual cycle

1.1.1 Architecture of the human uterus

The uterus is a thick-walled, muscular organ that is situated in the pelvic cavity between the bladder and the rectum (Gray and Lewis, 1918). Three layers constitute the uterus: an external layer called the perimetrium, a middle layer called the myometrium and an internal layer called the endometrium (Figure 1-1). The perimetrium is a serous coat which derives from the peritoneum; it covers the fundus and the whole of the intestinal surface of the uterus (Blackbum, 2014). It provides a relatively inelastic base upon which the myometrium develops tension. The myometrium represents the principal volume of the uterus. It consists of muscular fibres, arranged in layers, intermixed with blood vessels, lymphatic vessels, and nerves (Naftalin and Jurkovic, 2009). The myometrium is responsible for uterine contractions at the time of menstruation and during labour (Wilson and Worthen, 1979, Bulletti et al., 2000); it also provides a blood supply to the endometrium (Farrer-Brown et al., 1970, Rogers, 1996).

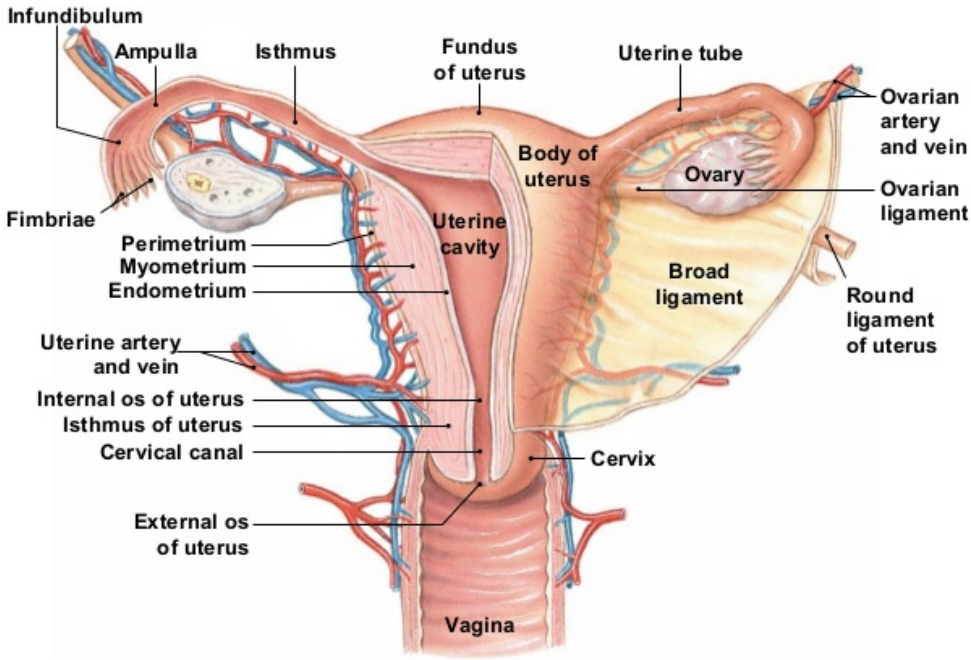

Figure 1-1 The anatomy of the human uterus.
Retrieved from Martini et al. (2013).

The endometrium is adherent to the myometrium, and is a multi-cellular tissue that undergoes monthly tissue remodelling in response to fluctuating levels of sex steroid hormones produced by the ovaries (reviewed in Critchley and Saunders (2009)). The endometrium has a luminal epithelium, which lines the uterine cavity and epithelial lined secretory glands as well as a heterogeneous stromal compartment. The endometrial stroma contains fibroblasts, blood vessels lined by endothelial cells, perivascular cells (pericytes and myofibroblasts) and a heterogeneous population of immune cells (reviewed in Evans and Salamonsen (2012b)). The secretory epithelium that covers the endometrial glands can extend to the endometrial-myometrial junction.

Two layers form the endometrial compartment: the functional and the basal endometrium. The functional layer is the region, which breaks down and sheds during menstruation; it is comprised of the luminal epithelium, sub-adjacent stroma and the luminal section of the secretory glands (Padykula et al., 1989). The region of the endometrium adjacent to the myometrium is the basal layer, this layer persists throughout the cycle and is believed to contain progenitor cells that reconstruct a new functional layer following menstruation or parturition (Gargett and Ye, 2012). The basal endometrium is represented by the mid-region of glands, which are surrounded by a compact stroma and the portion of the endometrium adjacent to the myometrium, where glands end and the endometrial-myometrial junction is located. The endometrial-myometrial junction, otherwise known as the inner myometrium, is defined as the zone between the endometrium, and the outer smooth muscle layer of the myometrium (Naftalin and Jurkovic, 2009). The inner myometrium is more highly vascularized than other regions of the muscle layer and the muscle fibres are also more densely packed (Tetlow et al., 1999).

1.1.2 Hormonal changes during the menstrual cycle

The endometrium plays an essential role in female fertility by acting as the tissue requires for establishing and successfully sustaining a pregnancy. In 1908, histological changes in the endometrium were first described by Hitschmann and Adler (1908), and half a century later a central dogma was postulated that changes in the cellular behaviour of the endometrium, including cellular proliferation, differentiation and apoptosis were strictly controlled by sex steroid hormones produced by the ovary

(Good and Moyer, 1968). This insight prompted Noyes et al. (1975) to precisely define the various histological changes of the endometrium in response to fluctuating levels of oestradiol and progesterone.

1.1.2.1 Hypothalamus-pituitary-ovary axis

The secretion of female hormones from the ovaries is controlled by endocrine signals and feedback loops within the hypothalamic-pituitary-ovarian axis as illustrated in Figure 1-2 (Yen, 1977). The signals that initiate secretion of ovarian hormones originate in the central nervous system, specifically from the hypothalamus, and subsequent secretion from the small endocrine organ found at the base of the brain, the pituitary.

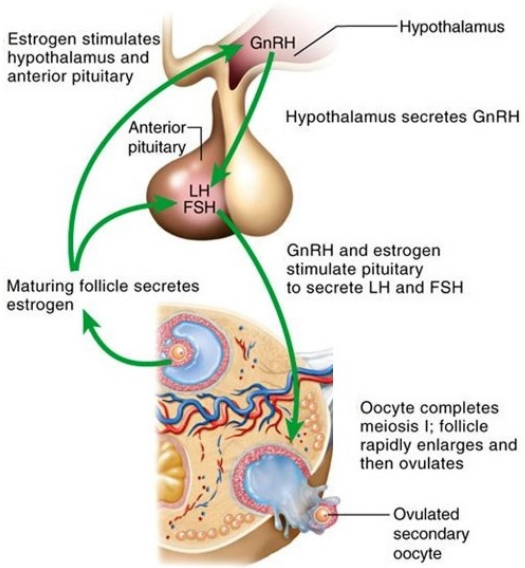

Figure 1-2 Schematic of hypothalamus-pituitary-ovariy axis.
Secretion of GnRH from the hypothalamus induces the production of LH and FSH hormones by the gonadotroph cells in the pituitary gland. LH and FSH are released into the circulation and signal to the theca and granulosa cells in the ovary to synthesise androgens, oestrogens and progesterone respectively.
Retrieved from Molina (2013).

The hypothalamus accounts for approximately 0.3% of the female adult brain however despite its size, it is densely packed with nuclei which are responsible for the homeostasis of several processes, such as endocrine regulation and reproduction (Mancall and Brock, 2011). The arcuate nucleus of the hypothalamus is where the production of gonadotropin releasing hormone (GnRH) takes place. GnRH is released in pulses into the pituitary circulation and after reaching the anterior pituitary induces the biosynthesis of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the anterior lobe of the pituitary and their release into the circulation (Beshay and Carr, 2013). FSH and LH act as endocrine hormones binding to their individual, specific, receptors, the proteins of which can be located in different somatic cells in

the ovary to stimulate steroidogenesis (detailed in section 1.1.2.2). The hypothalamus-pituitary-ovary axis is regulated by both positive and negative feedback loops involving ovarian hormones, as illustrated in Figure 1-2 (Moore and Price, 1932, Krsmanovic et al., 2009). In women, the effect is menstrual cycle phase dependent reflecting fluctuations in ovarian hormones. Briefly, the increase in circulating concentrations of oestrogens during the late follicular phases, as a result of biosynthesis in antral follicles (Britt et al., 2004), stimulates the production of GnRH from the hypothalamus and LH from the pituitary. As follicles mature, the granulosa cells in the follicles synthetize the aromatase enzyme in response to FSH. Oestrogens further increase the responsiveness of granulosa cells to FSH, which leads to the progression of the dominant follicle and a further increase in the amount of oestrogens secreted into the circulation (follicular phase for the ovary and proliferative phase for the endometrium). Ovulation occurs in response to a surge in LH and the granulosa cells transform into corpora luteal cells that secrete progesterone, which negatively regulates the axis both at hypothalamus and pituitary levels. If pregnancy is not established the corpus luteum regresses, oestrogen and progesterone concentrations fall precipitously, the negative feedback is removed and the cycle starts again (reviewed by Stocco et al. (2007)).

1.1.2.2 Ovarian steroid biosynthesis

Oestrogen and progesterone play key roles in the growth and differentiation of the human endometrium. In women, the main sources of circulating sex hormones are the ovaries and the adrenal glands. Predominantly, sex steroid biosynthesis occurs in the ovary, which contains by two endocrine structures: the follicular complex with granulosa and theca cells and the corpus luteum (Figure 1-3, Yen (1977)). The crucial functions of the ovaries are the formation of the oocytes and production of sex steroid hormones. The somatic cells surrounding the growing follicles produce oestrogens that feed back to the hypothalamus-pituitary axis, as described above, and also drive local follicle maturation (Lunenfeld et al., 1975). The gonadotrophins LH and FSH influence the ovary and trigger the steroidogenesis of testosterone, oestradiol and progesterone. Steroids are synthesised *de novo* from free circulating cholesterol (reviewed by Payne and Hales (2004) and Hu et al. (2010)), which derives from the liver and arrives in the theca cells of the ovary. The biosynthesis of oestrogen and

progesterone requires an enzymatic cascade in both theca and granulosa cells, detailed in Figure 1-3 (Short, 1962, Hillier et al., 1994). This is often referred to as the "two-cell, two gonadotrophin" mechanism, with LH binding to its cognate receptor (LHR) on the membrane of theca cells to trigger androgen synthesis, and FSH binding to the FHSR on the granulosa cells to stimulate the synthesis of the aromatase complex that converts testosterone into oestradiol.

Briefly, LH by binding the membrane bound G protein coupled receptor (GPCR), LHR, stimulates a second message signalling involving cyclic adenosine monophosphate (cAMP) (Marsh, 1976), which then induces the transcription of the *CYP11A* gene in the theca cells. The CYP11a enzyme plays a key role as it catalyses the conversion of cholesterol to pregnenolone (Chung et al., 1986). Androstenedione, a testosterone precursor, is synthetized via multiple intermediates including the enzymes of CYP17 (also known as 17α hydroxylase) and 17βHSD (*HSD17B*) (Patel et al., 2010).

FSH binds FSHR on granulosa cells, which is also a GPCR, and triggers a second messanger cascade involving production of cAMP to stimulate transcription of the CYP19 gene (aromatase) (Ryan et al., 1968). The aromatase enzyme complex converts androstenedione secreted from the theca cells, after cell membrane crossing, to oestrogens (oestrone and oestradiol), which are then released into the circulation and thereafter act in an endocrine manner on oestrogen-target tissues, including the endometrium.

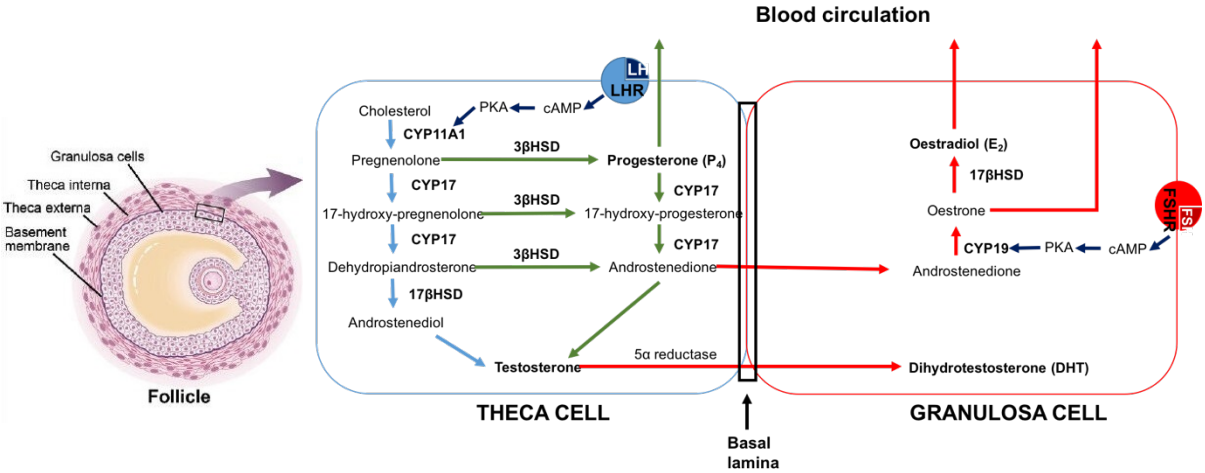

Figure 1-3 "Two cell-two gonadotropin" model.
Sex steroid hormones, oestrogen, progesterone and testosterone are synthesised in the somatic cells of the follicle, theca and granulosa cells. The synthesis is coordinated by the binding of LH and FSH to their GPCR receptors. Cholesterol is cleaved by CYP11A1 into pregnenolone, steroid dehydrogenase convert pregnenolone into progesterone (3βHSD) or into androstenediol (CYP17 and 17βHSD). Androstenediol can cross the basal membrane between the two cells, and is converted into oestrone and oestradiol, (CYP19 and 17βHSD) in the granulosa cells before being released into the blood stream. CYP11A1: cholesterol side chain cleavage cytochrome P450, CYP17: 17α hydroxylase/C_{17-20} lyase cytochrome P450, CYP19: aromatase, 17βHSD: 17β hydroxysteroid dehydrogenase, 3βHSD: 3β hydroxysteroid dehydrogenase, cAMP: adenosine 3',5'-cyclic monophosphate, PKA: protein kinase A, FSH: follicle stimulating hormone, FSHR: follicle stimulating hormone receptor, LH: luteinising hormone, LHR: luteinising hormone receptor. Adapted from Beshay and Carr (2013) and Doshi and Agarwal (2013).

The transformation of the follicles into corpora lutea changes the expression of steroidogenic enzymes so that progesterone, and not androgens, becomes the predominant hormone produced in the ovary during the secretory phase. This switch in steroid production is the result of the increased expression of CYP11A and 3βHSD favouring the conversion of cholesterol into pregnenolone and progesterone and a decrease in expression of CYP17 which reduces oestradiol production (Lachance et al., 1990).

1.1.3 Histological features of endometrium during the menstrual cycle

The human endometrium undergoes substantial transformation during every menstrual cycle with disintegration of the functional endometrium (menstrual phase), followed by its regeneration (proliferative phase) and differentiation of a new layer in preparation for an embryo implantation (secretory phase, Figure 1-4).

Based on the screening and histological staging of 8000 endometrial biopsies, Noyes et al. (1975) provided a classification of the different stages of the menstrual cycle which is a gold standard for histological staging. The length of the average menstrual cycle is 28 days, the convention numbers days of the cycle starting from menstruation (day 1) being followed by the proliferative phase (day 4 to 14) and after ovulation, the secretory phase (typically days 16 to 28). The secretory phase can be further subdivided into early (day 16-19), mid (day 20-24) and late secretory (day 25-28) phases. For a better understanding of the sequence of events in the human endometrium, the phases of the menstrual cycle are described in detail in the following sections.

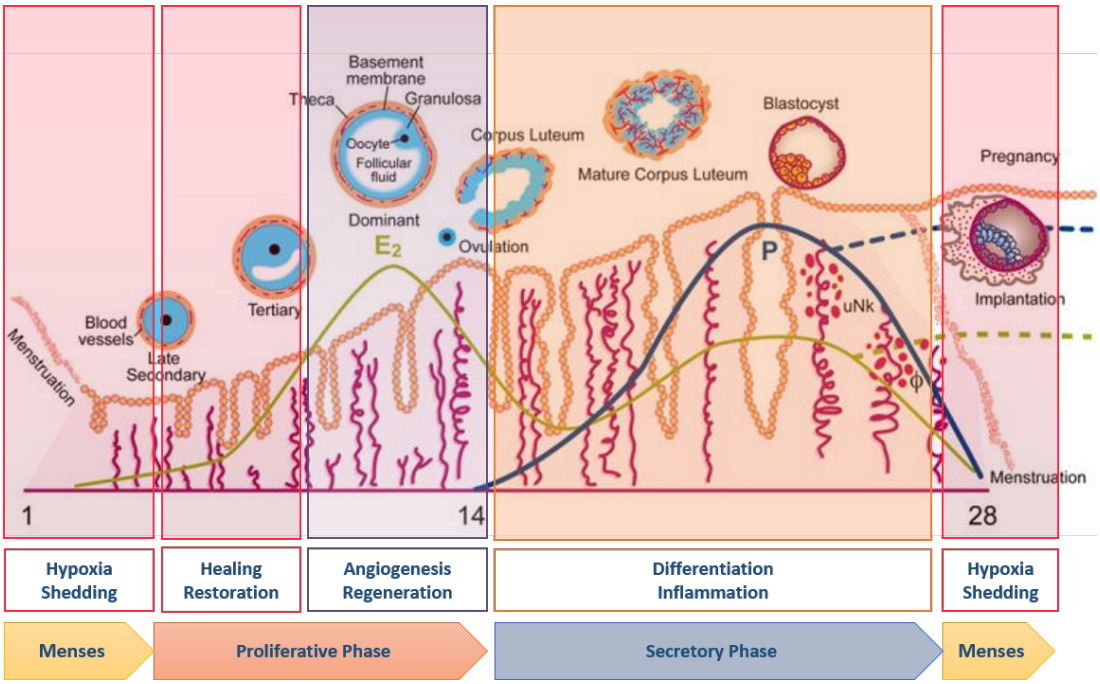

Figure 1-4 The human ovarian and functional endometrium during the menstrual cycle.
The fluctuating concentrations of E_2 and P_4 (P, progesterone) in blood are shown schematically. During the latter half of the cycle the numbers of tissue resident uterine natural killer cells (uNKs) and macrophages (ϕ) are increased. Image courtesy of Professor Saunders, originally drawn by Mr Pinner for the MRC Human Reproductive Sciences Unit.

1.1.3.1 The proliferative phase

During the proliferative phase (also known as follicular phase based of the activity of the ovary) the endometrium undergoes intense cellular proliferation as reported by Ferenczy et al. (1979) and Johannisson et al. (1987). Evidence of DNA synthesis and mitoses of multiple cells in the epithelium, stroma and vascular network has been described in samples recovered towards the end of the proliferative phase,

prior to ovulation (Talbi et al., 2006). The luminal epithelium initially appears thin after its regeneration post-menses but newly formed straight glands become more voluminous and tortuous as oestrogen concentrations rise (Figure 1-4). During the proliferative phase, the development of a glandular network occurs together with the formation of an elaborate vascular supply that is also accompanied by stromal cell proliferation (Gambino et al., 2002). Histologically, the endometrium thickens from about 2 mm recorded immediately after the menstrual phase, to 14 mm prior to ovulation on day 14, (see Figure 1-5, (Hess et al., 2006)). Notably, the presence of oestrogen receptor proteins in the endometrium are considered essential to facilitate normal proliferation and vascularisation. Specifically, studies using mouse models have shown that oestrogen regulates the proliferative phase through the oestrogen receptor isoform α, which induces stromal-epithelial interactions (Lubahn et al., 1993, Winuthayanon et al., 2010). The proliferative phase ensures the priming of the endometrium for the events necessary for hosting a pregnancy and it has been demonstrated that E_2 induces both the upregulation of the expression of both its own receptor (ERα) and also progesterone receptors (PR) (Couse and Korach, 1999) enabling to respond to progesterone released by the corpus luteum.

1.1.3.2 The secretory phase

After ovulation, the secretory phase begins (also called the luteal phase for the ovary, Figure 1-4). The transformation of the endometrium during the secretory phase is influenced by the high circulating concentrations of progesterone that are produced by the corpus luteum which induce both glandular and stromal cell differentiation. On days 15-16, the glandular epithelium starts to accumulate glycogen in its basal region, and the nuclei move towards the apical region of the cells (see Figure 1-5, (Hess et al., 2006)). By day 17, well-organized vacuoles are present in the glycogen granules and mitochondria have an increased size an adaptation which is thought to overcome of high demand for energy in the highly tortuous glands (Verma, 1983). On days 19 and 20, few vacuoles are left in the glandular epithelium as the glycoprotein-rich products are expelled by apocrine secretion into the glandular lumen. The uterine fluid found in the centre of the organ also contains plasma transudates derived from the endometrial vessels. The mid secretory changes become apparent after the day 20, with the stroma

become oedematous, due to an increase in vascular permeability (Okada et al., 2001). During the mid secretory phase there is also proliferation of epithelial cell and maturation/coiling of spiral arterioles in the functional layer.

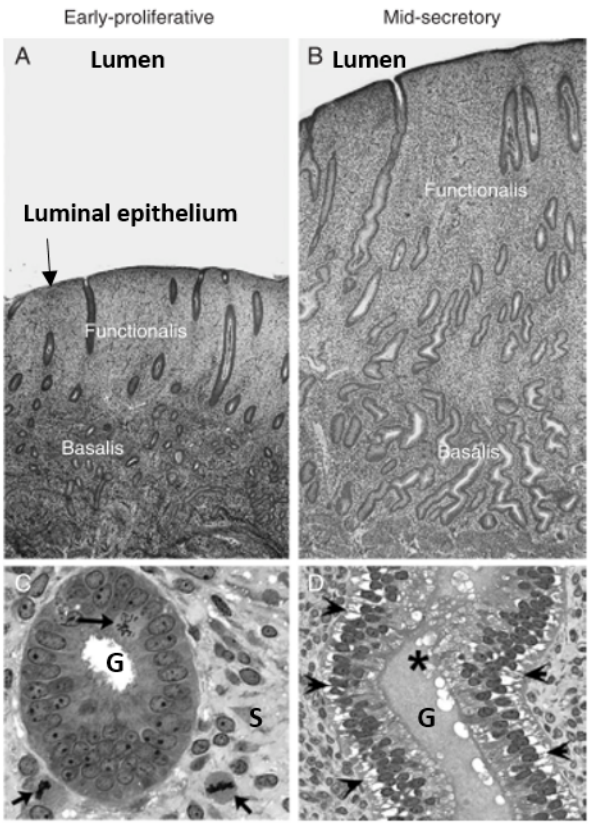

Figure 1-5 Endometrial thickness during the menstrual cycle.
A-B) Functional and basal compartments in full-thickness sections (endometrial-myometrial junction to luminal epithelium) during the proliferative **(A)** and the mid-secretory **(B)** phases. **C)** Mitotic events in glandular and stromal cells during proliferative phase (arrows). **D)** subnuclear vacuoles (arrowheads) and secretions in the lumen of glands (asterisk) during the mid-secretory phase. Image adapted by Hess et al. (2006). G: gland, S: stromal compartment.

Decidualisation, a specific cellular transformation event of stromal fibroblasts is initiated co-incident with maximum oedema (day 22) and is first observed adjacent to the blood vessels but thereafter proceeds in a "wave" to occupy the entire stromal compartment of the functional layer (Raine-Fenning et al., 2004). Decidualisation results in the conversion of spindle-shape fibroblasts into rounded epithelial-like cells with an enlarged cell surface (Gellersen and Brosens, 2014). At day 23, the fibroblasts are surrounded by spiral arterioles and capillaries. Stromal differentiation continues with sub-epithelial pre-decidua establishment (day 25), and by day 27 this appears as a solid sheet of pre-decidualised cells (Tang et al., 1993).

During days 24-25 of the menstrual cycle, there is a marked increase in lymphocyte infiltration into the endometrium (see section 1.2) which reaches its maximum by days 27-28 (late secretory phase) and persist after implantation in a

fertile cycle. If pregnancy occurs, the decidual cells will form the maternal component of the placenta, the basal decidua (Moffett and Loke, 2006). In the absence of the establishment of a pregnancy and consequent demise of the corpus luteum, levels of progesterone rapidly decline (progesterone withdrawal, Figure 1-4), which precipitously induces tissue breakdown with shedding that is experienced as menstruation (reviewed by Jabbour et al. (2009) and in Critchley and Saunders (2009)).

1.1.3.3 The menstrual phase

In absence of a developing blastocyst, the regression of the corpus luteum during the late secretory phase results in a rapid decrease in the circulating concentrations of ovarian-derived steroid hormones, and triggers a sequence of changes in endometrial tissue function that results in tissue breakdown and ultimately shedding of the functional layer of the tissue at menstruation.

Morphological evaluation of the tissue recovered at the time of menstruation has shown that it is characterized by cell death in the epithelium, with the exception of glandular epithelial cells located in the basal compartment, as well as blood vessel necrosis; focal haemorrhage and tissue shedding (Owen, 1975).

The cellular and molecular mechanisms that are initiated by progesterone withdrawal involve not only an impact on epithelial and stromal cells but also changes in the vascular compartment and the activity of immune cells (Maybin and Critchley, 2011). Notably menstruation shares many characteristics with an inflammatory response, including an increase in the biosynthesis of prostanoids, cytokines and chemokines (Jabbour et al., 2006).

It has been hypothesised that this inflammatory response can be considered as involving two phases: 1. An increase in the local concentrations of cytokines and prostaglandins in the endometrial tissue, induced in stromal cells in the response to the decreasing hormone concentrations (a progesterone receptor dependent event) (Jones et al., 2004, Catalano et al., 2007, Evans and Salamonsen, 2014); 2. An influx of immune cells in response to the local change in cytokines which is followed by immune cell activation (Kelly et al., 2001a, Kelly et al., 2001b).

1.1.3.4 Loss of vascular integrity

Spiral arterioles are unique structures in the endometrium of menstruating species. The classic studies by Markee using human endometrial tissue explants demonstrated that steroid (oestrogen/progesterone) withdrawal induced a pronounced vasoconstriction of the endometrial spiral arterioles for up to 24h (Markee, 1940). This vasoconstriction was then followed by the opposite vascular process, vasodilatation of the vessels, initiating an increase in blood flow. Ischemic damage induced by vasoconstriction facilitates the release of blood from the vessels to the luminal surface. The combination of the interruption of blood supply and the acute tissue haemorrhage, has been reported to be transient and thus similar to an "ischaemia-reperfusion" injury.

Several vasoactive mediators have been implicated in the response to steroid withdrawal. Prostaglandins (PGs) are locally produced and concentrations of vasoactive prostaglandin, PGE_2 have been reported to be higher than those of the vasoconstrictor $PGF_2\alpha$ at the time of menstruation (Baird et al., 1996b, Jabbour and Sales, 2004, Maybin et al., 2011). The vasodilator mediator nitric oxide (NO) is also locally synthesized in the endometrium and may have a role in regulating vascular tone (Cameron and Campbell, 1998), with vascular epithelial growth factor (VEGF) thought to have an effect on the induction of NO synthesis (Sharkey et al., 2000).

The vasoconstriction of arterioles may also cause a transient hypoxia in the functional endometrium. Hypoxia has been demonstrated in mouse model of menstruation using pimonidazole, which stains hypoxic cells (Cousins et al., 2016). The results obtained suggested that endometrial oxygen levels were reduced to 10 mmHg (110 mmHg is normal oxygen level) during menstrual phase (Fan et al., 2008, Cousins et al., 2016). Furthermore, the hypoxia-inducible factor 1 α (HIF-1α) protein, a transcription factor that is stabilised by low oxygen levels, was shown to be abundant in glandular and stromal cells in the functional endometrium during late secretory and menstrual phases, and absent during the proliferative phase (Punyadeera et al., 2006). HIF-1α activation has been shown to modulate the induction and/or repression of genes that regulate different cellular processes, such as angiogenesis, cell survival, proliferation and apoptosis (Piccoli et al., 2007) providing a link between hypoxia in the functional layer and processes required to restore tissue integrity.

1.1.3.5 Tissue breakdown

The endometrial extracellular matrix (ECM) is composed of collagen, laminin, gelatin, fibronectin, proteoglycans, and hyaluronic acid structures (Nair and Taylor, 2010). In addition to spiral arteriolar vasoconstriction, augmented ECM degradation is another principal mechanism that initiates menstruation. Metalloproteases (MMPs) control the degradation of the ECM (Gaide Chevronnay et al., 2012). Endometrial stroma expresses several MMPs (MMP-1, MMP-2, MMP-3, MMP-9, MMP-10), while the epithelium predominantly expresses MMP-7 (Salamonsen and Woolley, 1999, Salamonsen et al., 2000). The immune cell populations produce many other MMPs following their activation (Evans and Salamonsen, 2012a).

MMPs are generally secreted in an inactive form, as pro-MMPs. The synthesis of pro-MMPs increases just prior to menstruation in response to local signals (e.g. immune cell activation) as well as progesterone withdrawal. Withdrawal of progesterone leads to increased MMP production and activation of pro-MMPs triggering endometrial ECM degradation. The degradation of tissue matrix results in massive tissue destruction, loss of structural integrity, and vascular disruption. The endometrium is shed along with blood coming from the destroyed endometrial vasculature (Rogers and Abberton, 2003). Haemostasis is achieved after menstruation by coagulation in the basal endometrium (Davies and Kadir, 2012). Tissue breakdown induced by MMPs activates endometrial platelets and results in coagulation of the blood (Mastenbroek et al., 2015).

1.1.3.6 Re-epithelialization and tissue repair

The endometrium must rapidly repair to limit blood loss and prepare for arrival of a blastocyst in the following cycle. The processes implicated during an efficient endometrial repair share some features to classic wound healing, and these include inflammation, resolution, angiogenesis, tissue formation and tissue remodelling (Maybin and Critchley, 2015).

A remarkable study using human endometrial tissues reported that the “repair” of the functional layer occurs within the 48h from start of menses; therefore it is considered a rapid process (Ferenczy, 1976a). Scanning electron microscopy (SEM)

of endometrial samples during the menstrual phase (day 2), has documented a ragged and irregular surface with secretory glands together with a complete lack of an intact epithelial luminal layer (Ludwig and Spornitz, 1991). The regrowth of the epithelium, both glandular and luminal, starts before stromal compartment expansion, with epithelial cells proliferating from the necks of the glands to meet migrating cells from other glands, forming a new luminal surface (Garry et al., 2010). The restoration of the luminal surface with a fully reconstructed epithelial cell layer, is achieved by day 6 (Ferenczy, 1976a, Ferenczy, 1976b). A recent study employing SEM technology showed that the shedding of the functional endometrial layer appears to occur simultaneously with the repair process during menstruation, rather than in sequence as was once thought (Garry et al., 2009).

Notably, the initial re-epithelialization of the endometrium and active bleeding occur during in steroid-depleted environment, after regression of the corpus luteum and prior to significant oestrogen secretion from the growing follicles. Animal studies investigating the repair process have demonstrated that oestrogen is not required in the initiation of, or during the process of endometrial repair (Kaitu'u-Lino et al., 2007, Matsuura-Sawada et al., 2005).

1.1.4 Steroid hormones receptors

Sex steroid hormones, such as oestrogen and progestagens, have a critical impact on several physiological functions and are essential regulators of reproductive function.

Sex steroid hormones can alter cellular functions by binding to nuclear receptors, members of the nuclear receptor superfamily (Huang et al., 2010). The glucocorticoid receptor is also a member of the nuclear receptor superfamily (Rose et al., 2010). These steroid receptors all have a similar structure with a DNA-binding domain (DBD-C), which retains two zinc finger motifs, a hinge domain (D) and the receptor domain responsible for ligand binding (LBD-E) located towards the C terminus of the protein, as shown in Figure 1-6 (Griekspoor et al., 2007, Critchley and Saunders, 2009).

In humans, two isoforms of oestrogen receptor have been identified: ERα (*NR3A1*) and ERβ (*NR3A2*), which are encoded by two separate genes, *ESR1* and *ESR2* respectively, located on the human chromosomes 6q25.1 (*ESR1*) and 14q23.3 (*ESR2*) (Nilsson et al., 2001).

The human progesterone receptor (PR, *NR3C3*) is the product of a single gene, which is located on chromosome 11q22 (Rousseau-Merck et al., 1987). PR can exist as two different isoforms PR-A and PR-B, which differ from each other by the 165 N-terminal sequence of amino acids on the PR-B (Kastner et al., 1990) due to use of alternative start sites (Figure 1-6).

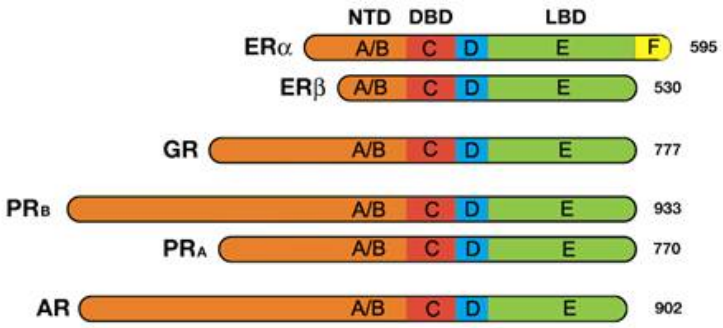

Figure 1-6 The steroid hormone receptor superfamily.
Steroid hormone receptors have a conserved structure constituted by a variable N-terminal domain (A/B), a common DNA binding domain (DBD), a hinge domain (D) and ligand binding domain (LBD-E) located toward the C-terminus. Adapted from Scally (2012).

The glucocorticoid receptor encoding gene (*NR3C1*) has been mapped onto human chromosome 5q31-32 (Francke and Foellmer, 1989). Two isoforms of GR have been described, GRα and GRβ, which are identical until amino acid 727 but then differ due alternative splicing. GRα has an additional 50 amino acids (total of 777 amino acids, represented in Figure 1-6) and GRβ an additional, non-homologous 15 amino acids (Oakley et al., 1996, Oakley et al., 1999). Only GRα appeared to be able to bind hormone and induce expression of a glucocorticoid-responsive reporters (Hollenberg et al., 1985, Taniguchi et al., 2010).

1.1.4.1 Steroid receptor signalling

According to the classical model of steroid hormone dependent gene activation, the receptors act as ligand activates transcription factors. In brief, steroids enter the cell, by either by passive diffusion through the lipid membrane or assisted by transporter proteins, and then bind to steroid receptors which are in the cytoplasm (Beato, 1989). Ligands bind in a "pocket" located in the LBD domain and this

provokes a conformational change in the receptor, which is accompanied by the dissociation of accessory proteins, exposing the DNA-binding domain (DBD) and sites for binding of cofactors typically in helix 12 at the C terminus of the proteins (McDonnell et al., 1993, Mangelsdorf et al., 1995, Losel and Wehling, 2003).

The genomic actions of steroid hormones may be mediated through different pathways, categorised in two main mechanisms: "direct binding" where the ligand-receptor complex directly binds to a specific DNA sequences in gene promoters or enhancers called steroid hormone response elements (oestrogen: ERE, progesterone: PRE and glucocorticoids: GRE), which are typically specific inverted palindromic sequences. Alternatively ligand-activated receptors use a "tethering" mechanism involving other DNA-bound transcription factors such as Jun, Fos and Sp1 and stabilises the DNA binding site of the specific factors (McDonnell et al., 1993) (Figure 1-7).

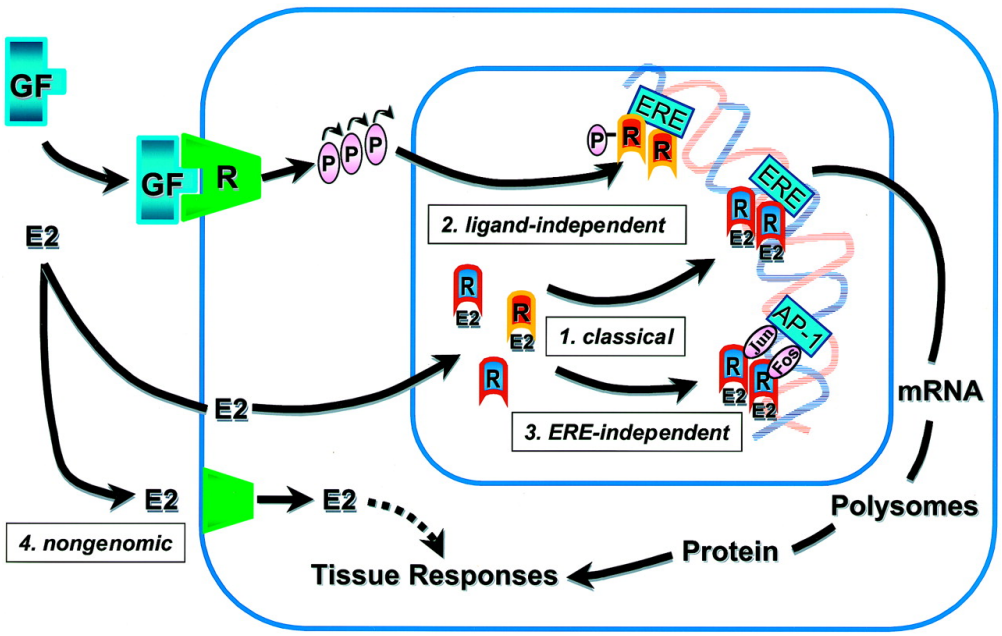

Figure 1-7 Different mechanisms of oestrogen receptor signalling.
The effects of E_2 can be mediated through four mechanisms: **1.** Classical ligand-dependent, E_2-ER complex binds to EREs in target promoters inducing an up- or downregulation of transcription and its consequent tissue responses. **2.** ligand independent pathway, growth factors (GF) or cyclic adenosine monophosphate (not shown) stimulate intracellular kinase pathways, leading to phosphorylation event (P) and activation of ER at ERE-containing promoters. **3.** ERE-independent, E_2-ER complex modifies gene transcription containing alternative response elements such as AP-1 through association with other DNA-bound transcription factors (Fos/Jun), which tether the activated ER to DNA, resulting in an up-regulation of gene expression. **4.** Cell-surface (non-genomic) signalling, E_2 activates a membrane associated binding site, that can be a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses (Hall et al., 2001).

In both cases, transcription depends upon recruitment of a cocktail of factors including polymerase II prior to activation or repression of transcription. The length of time between steroid entry and the accumulation of significant amounts of newly formed protein is in the order of hours, and the whole pathway is sensitive to particular inhibitors (Losel and Wehling, 2003).

In addition to the direct effect on transcription, steroid hormones have been shown to influence the activity of many other signalling pathways in a non-nuclear manner, by the binding to cell membrane receptors. This mechanism is usually referred to "non-genomic" and secondary messenger mediated, involving protein kinase cascade (e.g. MAPK) (Migliaccio et al., 1996, Clark and Lasa, 2003, Treviño et al., 2013). Furthermore, steroid hormones are also reported to act via members of the G protein coupled receptors superfamily (Kelly and Wagner, 1999) and exert rapid effects with increase of cAMP (Aronica et al., 1994), lipase or kinase pathways and/or with ion channel (Tesarik and Mendoza, 1995), inducing an alteration of intracellular Ca^{2+} (Figure 1-8).

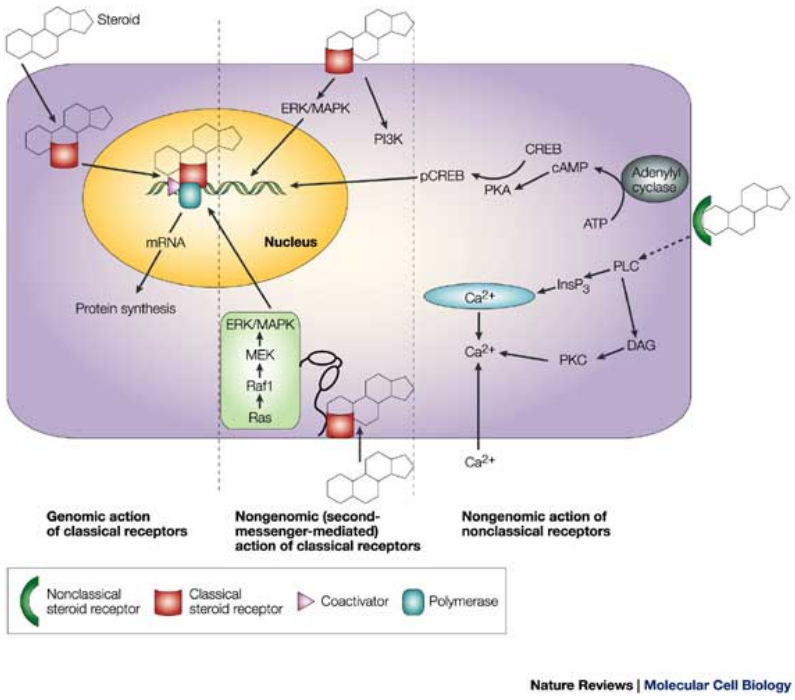

Figure 1-8 Simplified schemat of steroid hormone actions through the different pathways.
This image represents the possible effects of steroid hormone-receptor mechanisms, via the "classical" genomic action (left part) or non-genomic ways: via conventional steroid hormone membrane receptors (middle part) or via non-classical receptors (right part). Image retrieved from Losel and Wehling (2003).

Oestradiol and oestrone are the classical endogenous ligands for both ERα and ERβ, and both exhibit agonist activity at ERs (Gruber et al., 2002). Other molecules have been synthesised which show specific binding to ERs, for example two molecules have been widely used as specific agonists for studies in cell lines and mice, propyl-pyrazole triol (PPT; ERα specific) and diarylpriopionitrile (DPN; ERβ specific). Specific oestrogen receptor modulators (SERMs) are compounds capable of mixed agonist and antagonist actions on ERs, with their tissue specific actions dependent on the local availability of co-factors in different cells/tissues (Bryant, 2001, Sun et al., 2002). Probably the most widely used SERM is tamoxifen, it is commonly employed as ER antagonist in breast cancer therapy, but it acts as an agonist in endometrial tissue (Dutertre and Smith, 2000, Katzenellenbogen et al., 2000). The compound ICI 182,780 (fulvestrant) acts as full antagonist for both ER isoforms by inducing receptor turnover (Osborne et al., 2004).

Progesterone receptors (PRs) may be activated, similar to ERs, by the endogenous steroid progesterone (P_4), as well by the synthetic compounds with agonist or antagonist activity; medroxyprogesterone acetate is one of the most widely used PR agonists (Sitruk-Ware, 2004). Examples of SPRMs (selective progesterone receptor modulators) are ulipristal and asoprisnil, which exert different actions as agonist or antagonist in a tissue dependent manner (reviewed by Wagenfeld et al. (2016)).

Glucocorticoid receptors (GRs) can be activated by both by cortisol from the circulation, cortisol generated locally within tissues by the actions of HSD enzymes (hydroxysteroid dehydrogenase) and by synthetic steroids such as prednisolone and RU 24858 (Vayssiere et al., 1997, McMaster and Rays, 2008), as well as by non-steroidal molecules, as ZK 216348 which can act as selective glucocorticoid receptor modulators (SGRM) (McMaster and Rays, 2008).

1.1.4.2 Steroid receptor expression in the human uterus

The expression of steroid hormone receptors is tightly regulated both temporally and spatially in the uterus (reviewed in Critchley and Saunders (2009) and Gibson and Saunders (2012)).

Oestrogen receptors are present in cells within all the different uterine compartments including the myometrium and both the basal and functional endometrium. In the myometrium, mRNA concentrations of ERα are higher than ERβ, but a switch in the ratio of mRNAs has been reported in the myometrium of postmenopausal women (Jakimiuk et al., 2004). In reproductive age women the ratio of ERα to ERβ in the endometrium varies accordingly to the levels of circulating hormone during the menstrual cycle. During the oestrogen dominated proliferative phase expression of ERα is reported to be high in the epithelial glands and in the stroma but has not been detected in endothelial cells lining the blood vessels (Critchley et al., 2001). The protein decreases in the glands and stromal cells after the rise of progesterone levels during the progesterone dominant secretory phase (Critchley et al., 2002). ERβ is predominantly present during the secretory phase, and is notably expressed by stromal, epithelial and endothelial cells in the functional layer of the endometrium (Critchley et al., 2001). Furthermore, the action of oestrogen on endometrial endothelial cells (HEECs) has been reported to be ERβ-dependent (Greaves et al., 2013). Notably most studies on endometrial leukocyte populations, including those focusing on natural killer cells (uNKs) and macrophages has reported they do not express ERα and are immunopositive for ERβ (Hunt et al., 1998, Henderson et al., 2003, Thiruchelvam et al., 2013).

Progesterone receptor expression varies throughout the phases of the menstrual cycle (Ingamells et al., 1996) and is reported to be oestrogen regulated with an increase in PR protein expression being detected in the endometrium during the proliferative phase (Mote et al., 2000). PR-A is the predominant type in the stromal compartment during the secretory phase and early pregnancy (Wang et al., 1998). While the expression of both isoforms PR-A and PR-B is drastically reduced in the glandular epithelium during the secretory phase, it has been shown that PR-A expression is persistent in the stroma and in particular in stromal cells associated with blood vessels, but that PR is no detectable in the endothelium (Perrot-Applanat et al., 1994, Critchley et al., 2001). The expression of PR in the basal endometrium is not affected by the hormonal changes, and stromal and epithelial cells are both immunopositive for PR throughout the cycle (Snijders et al., 1992).

Glucocorticoid receptor can be detected in the nuclei of endometrial stromal cells in all phases of the menstrual cycle, while epithelial cells are negative (Bamberger et al., 2001, Henderson et al., 2003). The impact of glucocorticoids is regulated not only by its receptor expression, but also by the presence of steroid-metabolizing enzymes (which are responsible for ligand bioavailability). The 11b-HSD family converts cortisone (inactive glucocorticoid form) into cortisol (active form) by the 11b-HSD1 enzyme and inactivates cortisol into cortisone by 11b-HSD2 (Jabbour et al., 2006). It has been demonstrated that higher concentrations of 11b-HSD2 are present in the endometrial stroma throughout the phases of the cycle, compared to 11b-HSD1 and 11b-HSD2 expression rises during the secretory phase in epithelial cells (McDonald and Henderson, 2006). It was also confirmed by Kuroda et al. (2013) that the increase of mRNA expression of 11b-HSD1 is driven by progesterone in decidualizing human endometrial stromal cells (HESCs). Notably, GR expression was also detected in some of the endometrial leukocytes, including the uNKs (Henderson et al., 2003) and macrophages (Thiruchelvam et al., 2013).

1.2 Leukocytes in endometrium

The immune cell complement of the human endometrium is dynamic, and varies almost every day in response to the hormonal changes occurring during the normal menstrual cycle and throughout pregnancy (Jabbour et al., 2006). Inflammatory cells constitute a substantial proportion of the total cell population within the endometrial stroma with CD45 (leukocyte common antigen) positive cells being located throughout the endometrium in both basal and functional layers (Kammerer et al., 2004).

It has been reported that immune cell numbers change over the different phases of the cycle, representing 10-15% of cells in the proliferative phase, 20-25% of cells in the late secretory phase, and up to 40% of cells in the menstrual phase, detailed in Figure 1-9 (Kamat and Isaacson, 1987, Salamonsen and Woolley, 1999, Maybin and Critchley, 2015). This population mainly consists of uterine natural killers (uNKs), macrophages, neutrophils, eosinophils and mast cells (Salamonsen and Lathbury, 2000). Temporally, the proportion of different immune cell subtypes in the endometrium varies. For example, following the transition from the proliferative to

secretory phase, high numbers of uNKs and macrophages are detected (Kammerer et al., 2004). Whilst neutrophils and eosinophils are absent during most of the menstrual cycle, immediately before menstruation there is a stricking increase in their numbers in the functional endometrium (Jeziorska et al., 1995, Salamonsen and Woolley, 1999). In contrast some immune cells do not appear to fluctuate during the cycle, and this includes mast cells. Uterine leukocytes are described in detail in the sections below and as mast cells are the main focus of this thesis, they are reviewed in greatest detailed in section 1.3.

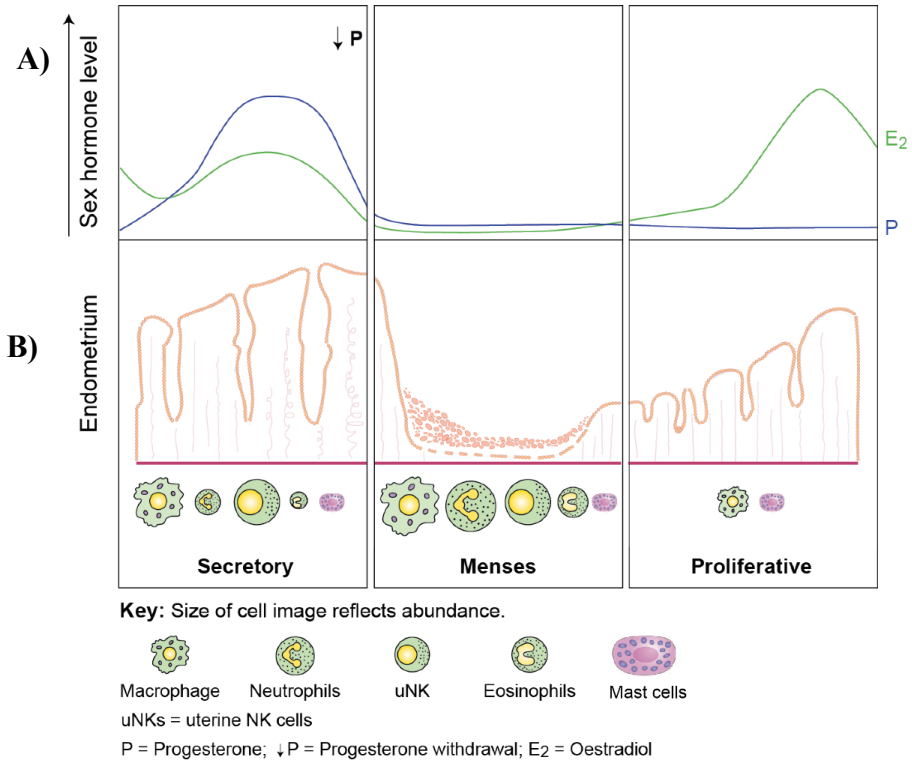

Figure 1-9 The human leukocytes during the menstrual cycle.
A) Sex steroid oscillations during the luteo-follicular transition. **B)** Overview of the uterine leukocyte population during the menstrual cycle. The size in panel **B** reflects the abundance in the endometrium. Image adapted from Maybin and Critchley (2015).

1.2.1 Uterine natural killer cells

The most abundant subtype of $CD45^+$ cells in the endometrium are uterine NK cells (uNKs). Although they are all but absent during the early stages of the menstrual cycle, uNKs comprise over 70% of the endometrial leukocytes during receptive phase when implantation in favoured, a time in the cycle that is coincident with elevated circulating progesterone levels and decidualisation of stromal fibroblasts (Bulmer et al., 1991).

The increase in uNK cell number has been attributated both to local NK cell proliferation within the endometrium and also to an additional influx of circulating NK cells (Jones et al., 1997, Chantakru et al., 2002). NKs specific to the endometrium, defined as uterine NKs (uNKs), are phenotypically different from the peripheral blood NK population (pbNKs). Up to 90% of pbNKs are phenotyped as $CD56^{dim}$-$CD16^{pos}$ while the minority is $CD56^{bright}$-$CD16^{neg}$ (Koopman et al., 2003). uNKs appear to be exclusively $CD56^{bright}$-$CD16^{neg}$ and are relatively granular, a feature not shared with the small percentage of $CD56^{bright}$-$CD16^{neg}$ pbNKs. There are also important functional differences between endometrial and peripheral NK cells, for example uNKs show a relatively weak cytotoxicity and increased cytokine secretion compared to pbNKs (Cooper et al., 2001).

During the late secretory phase and coincident with decidualisation, numbers of uNKs greatly increase (Bulmer et al., 1991). Unlike pbNKs, uNKs can secrete an array of factors with angiogenic actions, such as vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and angiopoetin 2 (Li et al., 2001, Hanna et al., 2006, Lash et al., 2006). Notably, uNKs are often located near the spiral arterioles and accumulate in perivascular regions to exert their angiogenic action.

It has been postulated that the function of uNKs could be influenced by sex hormones, due to their phase specific influx. Studies in our group have demonstrated that uNK cells immunonegative for ERα and PR (Henderson et al., 2003) and immunopositive for ERβ and GR (Henderson et al., 2003, Bombail et al., 2008).

1.2.2 Macrophages

Monocytes develop from a common myeloid progenitors found in the bone marrow or the splenic reservoir (Hettinger et al., 2013). Progenitors are released into the circulation and subsequentially recruited into tissues, where differentiation into mature macrophages takes place. Tissue-resident macrophages show phenotypical differences based on their specific tissue environment (Radzun, 2015). Macrophages exert precise roles in the immune response through phagocytosis of apoptotic bodies, or modulation of other immune cells through the secretion of inflammatory factors in the tissue (Wynn and Barron, 2010, Thiruchelvam et al., 2013, Zhou et al., 2016).

Macrophages are important members of the immune cells in the endometrium and their number is tightly regulated temporally and spatially during the menstrual cycle. Macrophages make up 1-2% of the stromal endometrial compartment during the proliferative phase, their number increasing to 3-5% after the surge of progesterone in the secretory phase, and reaching their peak after progesterone withdrawal (late secretory) and menses, accounting for approximately 6-15% of the total stromal cells in the tissue (Kamat and Isaacson, 1987, Salamonsen and Woolley, 1999, Salamonsen et al., 2002, Thiruchelvam et al., 2013).

It has been demonstrated that endometrial macrophages are able to release MMP-9, MMP-12, MMP-14 and plasminogen activator, which are all molecules implicated in the breakdown of the functional layer of the endometrium (Jeziorska et al., 1996, Salamonsen and Woolley, 1999, Curry and Osteen, 2003).

Similar to uNKs, endometrial macrophages appear to express ERβ and GR, but not PR (Hunt et al., 1998). There is also evidence to suggest that oestrogen can influence macrophages; the administration of exogenous oestrogen to women undergoing "*in vitro* fertilization" treatments induced a significant increase of macrophages in the functional endometrium (DeLoia et al., 2002). *In vitro* culture systems have been used to show macrophages also can respond to cortisol, promoting release of factors that promote angiogenesis (Thiruchelvam et al., 2016).

1.2.3 Neutrophils

Neutrophils are the most abundant leukocyte in the human immune system; in normal tissues they have a short lifespan but in inflamed tissues their lifespan can increased, as their rate of apoptosis is reduced by pro-inflammatory mediators and hypoxia (Ward et al., 1999, Cross et al., 2006). Neutrophils are released in the blood stream as mature or nearly mature immune cells (Mary, 1985, Galli et al., 2011). Neutrophils contain specific secretory granules, which store a diverse range of molecules that differ with the stage of neutrophil maturation.

Neutrophils are closely associated with tissue damage in several immune disorders (Leitch et al., 2008, Wright et al., 2010, Robb et al., 2016). Neutrophils have been localized in the human endometrium, by staining for neutrophil elastase.

Endometrial neutrophils have also been phenotyped as $CD11b^{pos}$, $CD66b^{pos}$ and $CD16^{pos}$ (Yeaman et al., 1998). Once activated they can release an array of pro-inflammatory cytokines such neutrophil-elastase, interleukin 8 (IL-8) and tumour necrosis factor α (TNFα) (Li and Verma, 2002).

Endometrial neutrophils are almost undetectable during most of the menstrual cycle, however their number drastically increases on days 27-28 of the late secretory phase, when they can account for up to 6-15% of the total number of endometrial stromal cells (Salamonsen and Woolley, 1999). Furthermore, studies on neutrophils in a mouse model of menstruation suggested that they were involved in the resolution of the endometrial wound as their depletion with antibody against Ly6G induces a delay in endometrial repair (Kaitu'u-Lino et al., 2007).

Neutrophils store high levels of MMPs in their granules and have the ability to activate resident MMPs of the endometrium to then initiate tissue breakdown (Gaide Chevronnay et al., 2012, Maybin and Critchley, 2015). There is evidence that neutrophils may respond to hormonal changes, as they are reported to express both isoforms of ER (α and β), PR and GR (Strickland et al., 2001, Molero et al., 2002, Bhattacharjee et al., 2012, Bhattacharjee et al., 2014).

1.2.4 Eosinophils

Eosinophils are members of the leukocyte population resident in mucosal tissues; they are considered multifunctional immune cells that contribute to various physiological and pathological mechanisms. Eosinophils derive from a common progenitor, which is capable of generating colonies of either eosinophils or basophils (Kita, 2011); mature eosinophils are present in the peripheral circulation. Eosinophils are a source of both regulatory and pro-inflammatory cytokines and chemokines (Hogan et al., 2008). Immunolocalization of the eosinophil specific cationic proteins (ECP1 and ECP2) has identified eosinophils in the human endometrium. Like neutrophils, eosinophils have been reported to be absent in the endometrium during day 5-26 of the menstrual cycle (proliferative and secretory phases), and there was an influx at day 26, which last until day 28 (Jeziorska et al., 1995). Extracellular staining for ECPs shows that eosinophils in the endometrium appear to be activated (Jeziorska

et al., 1995). Eosinophils also contain VEGF and MMPs in their granules (Okada et al., 1997, Puxeddu et al., 2005), suggesting they might complement other immune cells playing a role in tissue remodelling.

There is evidence that eosinophils respond to oestrogenic stimuli (Douin-Echinard et al., 2011). Steroid hormone receptor expression has been investigated in human uterine eosinophils and immunopositivity for both isoforms of ER and GR has been reported (Tchernitchin et al., 1974, Tchernitchin et al., 1976, Peterson et al., 1981), but they appear immunonegative for PR (Aerts et al., 2002).

1.3 Mast cells

MCs have long fascinated the scientific and medical communities due to their because of their association with the pathophysiology of common conditions such as asthma, dermatitis and allergic reactions. MCs were first identified by Ehrlich in the late XIX century (Ehrlich, 1878). They exhibit an array of adhesion molecules and immune response receptors that confer an advanced capability to react to multiple specific and nonspecific stimuli (Metcalfe et al., 1997). MCs are often detected in body sites that are in contact with the external environment and are play a role in host defence. The extensive tissue distribution of MCs supports their potential not only to act as first responders during host defence, but also to react to environmental alterations and communicate to other cells. Evidence that MCs are involved in tissue repair, wound healing and angiogenesis also support the crucial role they play in adaptive immunity (da Silva et al., 2014). MCs can rapidly interact with, and respond to, their surrounding environment and when activated they release a whole array of biologically active mediators and therefore even a small perturbation of the delicate balance between MC and their environment may result in devastating effects to the organism (Moon et al., 2010, Galli et al., 2011).

1.3.1 Growth, development and survival

MCs develop from $CD34^{+}/CD117^{+}$ pluripotent progenitors originating from the bone marrow (Kirshenbaum et al., 1999). The progression of progenitors cells to fully mature MCs is mainly dependent on c-kit receptor activation (CD117), which occurs as a consequence of the action of stem cell factor (SCF) which stimulates in receptor

dimerization and auto-phosphorylation (Gilfillan and Tkaczyk, 2006). Modifications of the c-kit receptor found on progenitors, as in the $Kit^{W\text{-}sh/W\text{-}sh}$ "sash" mouse, leads to a reduced number of MCs (Tsai et al., 2011).

Committed $CD34^{+}$ progenitors are released into the circulation, from where they can migrate into tissues throughout the body. The progenitors become terminally differentiated into mature MCs with tissue-specific phenotypes due to the influence of cytokines present in the tissue microenvironment (Metcalfe et al., 1997). The migration of MC progenitors appears to be controlled in a tissue specific manner and it has been reported to differ between tissue such as the intestine and the lungs (Fox et al., 1988). The signals that might influence the uterine specific trafficking of MC progenitors are not yet known. Generally, specific membrane proteins are required to facilitate MC progenitor trafficking including integrin α4β7 and CXCR2 found on their surface, and VCAM-1 which is present on the endothelial cells (Gilfillan et al., 2011). Evidence for the importance of these factors has been obtained from studies in integrin β7 null mice and studies using an antibody that blocks VCAM-1. Both strategies resulting in a profound deficit in the population of both MC progenitors and mature MCs (Artis et al., 2000, Gurish et al., 2001, Abonia et al., 2002). A similar effect has also been reported in a CXCR2 deficient mouse (Abonia et al., 2005). These studies highlight the importance of the IL-8-CXR2 pathways in MC recruitment. Trafficking from the capillaries to the peripheral tissues is maintained during homeostasis, but is drastically increased during inflammatory states (Haberstroh et al., 2002, Rose et al., 2003, Deshmane et al., 2009), providing a mechanism to explain the increased number of MCs at the sites on inflammation (Halova et al., 2012)

Fully differentiated tissue resident MCs are long-lived cells, but their lifespan is dependent on the maintenance of concentrations of SCF in the surrounding tissue. Accordingly, a reduction of SCF induces human mast cells to undergo apoptosis (Jensen et al., 2008). Moreover, SCF induced MC survival has been shown by Ekoff and Nilsson (2011) to be related to inactivation of FOXO3a, followed by the downregulation of the pro-apoptotic BH3-only protein.

1.3.2 Human and murine MC subtypes

MC maturation has been demonstrated to be environment specific, and the concentrations of different growth factors and cytokines can influence the progenitor-MC differentiation and the final mature MC phenotype (Jamur and Oliver, 2011). Accordingly, the MC populations within an individual may exhibit a high degree of heterogeneity due to their widespread tissue distribution.

Enerback (1966a, 1966b) was the first scientist to demonstrate the existence of two rodent MC subtypes: the mucosal MC (MMCs) and the connective MC (CTMCs). In the mouse the MMCs have been identified in the mucosal epithelium of the lung and gastrointestinal tract (Irani and Schwartz, 1989). MMC specific protease content is characterized by mast cell protease 1 and 2, mMCP-1 and mMPC-2, both of which are chymases. CTMCs are identified in the intestinal submucosa, peritoneum and skin, and contain granules that have mMCP-4 (chymase) as well as mMCP-6 and mMCP-7, both of which are tryptases and carboxypeptidase A (mCPA). Notably, the appearance of murine MCs as revealed by toluidine staining differs depending on this subtype-specific granule structure (Irani and Schwartz, 1989) (see Figure 1-10).

The chymase-like (chymase) protease family expressed in rodents differs from that in humans in terms of function. For example, MCP-5 presents a catalytic domain mutation that fundamentally changes its specificity, from chymotryptic to elastolytic

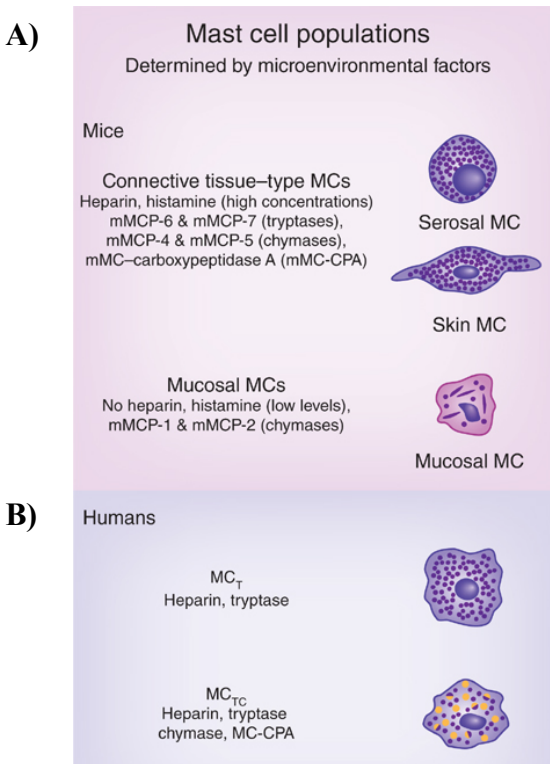

Figure 1-10 Murine and human MC subtypes. **A)** Murine MCs can be categorized into two subtypes based on their anatomical location, as being either connective or mucosal. The murine MC types differ in their granule structure and its components. **B)** Human MC subtypes are predominantly of two types: MC_T and MC_{TC} based on their protease granule content (Galli et al., 2011). A third rare subtype MC_C is not illustrated in this figure.

(Kunori et al., 2002, Karlson et al., 2003).. MCP-4 instead is the most similar to human chymase in terms of target preferences and expression pattern (Caughey, 2007a).

MMC granules are constituted by chondroitin sulfate chains of serglycin proteoglycans, whereas CTMC granules are formed by heparin chains of serglycin proteoglycans, as detailed in Figure 1-12 (Yurt et al., 1977, Enerback et al., 1986, Metcalfe et al., 1997). Therefore, they present with a different metachromatic staining with toluidine blue. As reported by Enerback (1966a), CTMCs appeared intensively purple stained whereas MMCs are only weakly stained, as illustrated in Figure 1-10-A. Another difference between the two murine MC subtypes is the content of histamine and lipid mediators, as only CTMCs are able secrete histamine and prostaglandin 2 upon stimulation (Heavey et al., 1988).

Human MCs are classified based on the expression of the proteases in their granules (Figure 1-10), the most common subtype are MC_T containing tryptase/heparin. There are also MC_{TC} which contain both tryptase and chymase, and a rare and less studied subtype MC_C, which is reported to be immunopositive for chymase but not tryptase (Irani et al., 1986, Irani and Schwartz, 1989, Weidner and Austen, 1993). MC_T are predominant in the intestinal and pulmonary mucosa, similar to MMCs, and MC_{TC} are located in the skin, lung and gastrointestinal mucosa, paralleling the locations of CTMCs (Irani et al., 1986). MC_C have been described in bronchi and gastrointestinal submucosa and mucosa (Weidner and Austen, 1993). Human MC granule structure does not changes between the three subtypes, as they all rely on a structure of heparin and chondroitin sulphate proteoglycans, at a 2:1 ratio (Metcalfe et al., 1979, Thompson et al., 1988).

The tissue specificity mast cell subsets and their protease content, brings into consideration the presence of specific inhibitors and target substrates for the proteinases, which also may vary from tissue to tissue. Thereby, it has been shown that proteinases released by MMC within gut epithelium during nematode infection (Tuohy et al., 1990) are initially likely to encounter lower concentrations of plasma-derived proteinase inhibitors such serpins (Irvine et al., 1990), when compared with CTMC, which are often located in the proximity of small blood vessels. Thus, the plasma

extravasation, rich in inhibitors, would promptly interfere with the chymases, released by CTMC, but this effect would be retarded in the epithelium, where the inhibitors need to diffuse into the tissue to act on MMCs. Therefore it has been postulated that the *in vivo* extracellular functions of mast cell specific proteinases is driven by: the specificity of the proteinase; the efficacy of inhibition and the ratio of proteinase/inhibitor; the solubility and stability of the proteinase itself; and the accessibility of target substrates and their susceptibility to proteolysis (Miller and Pemberton, 2002).

MCs represent a small percentage of the endometrial leukocyte population, which is reported to be relatively constant through the menstrual cycle. MCs have been identified in all layers of the uterus (Sivridis et al., 2001). Both MC_{TC} and MC_T subtypes have been identified in the human uterus (Jeziorska et al., 1995). MCs in the functional layer are reported to have a low granule content during the premenstrual stages which may suggest they are activated (Jeziorska et al., 1995).

MCs have been implicated in the active biochemical process of extracellular matrix (ECM) degradation that occurs during menstruation and because they are able to release both precursors of metalloproteinases (MMP-2, MMP-9) and collagenase (Lees et al., 1994, Milne et al., 2001). Moreover, MCs may be involved in implantation due to their ability to secrete histamine, which alters vascular permeability and induces stromal decidualisation (Wordinger et al., 1986, Menzies et al., 2011). Some mediators released by MCs such as histamine and serotonin have been shown to be associated with myometrial contractions, as they act as oxytocic agents (Menzies et al., 2012, Cruz et al., 1989).

Due to the activation of uterine MCs throughout the different phases of the menstrual cycle, it has been hypothesised that hormones might be involved in inducing MC degranulation. In non-reproductive tissues and the HMC-1 cell line, MCs are reported to express ERα and PR, but appear to be immunonegative for ERβ (Pang et al., 1995, Zhao et al., 2001, Nicovani and Rudolph, 2002, Zaitsu et al., 2007, Jensen et al., 2010). Investigations on GR expression using rat-derived MC lines, showed the expression of GR, both isoforms, on MC membranes (Oppong et al., 2013, Oppong et

al., 2014). To date there is no detailed phenotypic information related to steroid hormone receptors in MCs resident in the endometrium.

1.3.3 MC granules

The most distinguishing morphological characteristic of MCs is their high content of electron-dense lysosome-like secretory granules that represent the major component of their cytoplasm (as shown in Figure 1-11). In fact, it was the presence of multiple secretory granules that drew the attention of Paul Ehrlich, resulting in their naming as "mastung" cells, that in German means "well fed" (Ehrlich, 1878). Identification of MCs has usually been achieved by employing various cationic dyes, such toluidine blue.

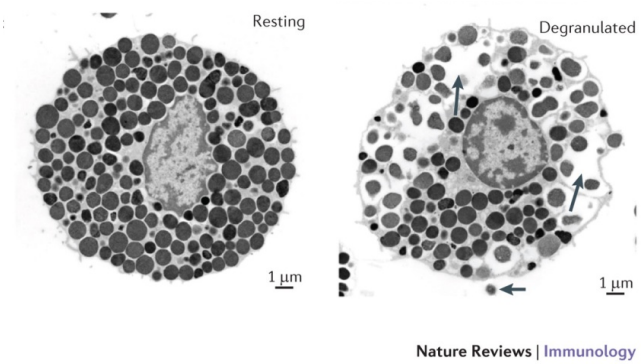

Figure 1-11 MC granule morphology **A)** Mature granules contain electron-dense material, which are equally distributed in the cytoplasm. B) Degranulating MC, with "degranulation channels" (long arrows), Figure adapted from Wernesson and Pejler (2014).

The serglycin proteoglycans are formed by a core protein to which chains of polysaccharide are covalently attached through glycosidic bonds to the serine residues of the core site (Wernersson and Pejler, 2014). The glycosidic part can be composed of heparin or chondroitin sulphate, both assembled in disaccharide chains (Figure 1-12). They provide a mesh structure that enables the storage of molecules in the granules (Ronnberg et al., 2012).

In addition to the storage, it has been shown that MC proteoglycans may have an effect on the activities of MC proteases. For examples, it has been demonstrated that tryptase's stability is highly dependent on the presence of heparin that acts by

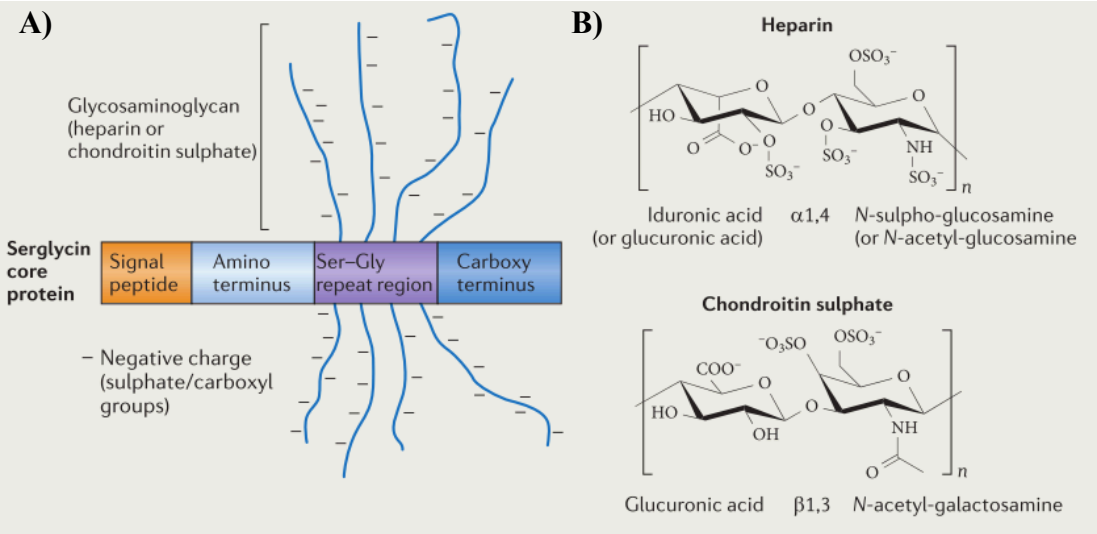

Figure 1-12 MC proteoglycan types and structure.
A) Proteoglycans are comprised of a "core protein" on which the glycosamiglycan (GAG) chains are covalently bound to serine residues (purple segment). **B)** Two different types of GAGs, heparin with chains of iduronic/glucuronic acid–glucosamine disaccharide (top), or chondroitin sulphate, are formed from glucuronic acid–N-acetyl-galactosamine disaccharide repeats (Wernersson and Pejler, 2014).

crosslinking the individual tryptase monomers and stabilizes the active tetramer of the protease (Hallgren and Pejler, 2006). On the contrary, the association of chymase with heparin chains has been shown to induce an inactivation of chymase substrates (Pejler et al., 2007). Therefore, heparin GAGs are present and they are required for protecting tryptase activity in CTMCs, where tryptase is the most abundant protease, and not in MMCs.

MCs produce and store three main classes of molecules: pre-formed granule-associated mediators, newly generated lipid mediators, and a variety of cytokines and chemokines, which are listed in detail in Table 1-1. Among these preformed/prepacked compounds, histamine, together with serotonin are the most prominent biogenic amines found in the MC granules. In line with the lysosome-like proprieties of granules (Arvan and Castle, 1998), MC granules also contain several lysosomal hydrolases as well as cytokines, such as TNFα and factors that regulate angiogenesis such as VEGF (Wernersson and Pejler, 2014). But notably, the major constituents of granules are the MC specific serine proteases: tryptase and chymase (Pejler et al., 2007). Other proteases such as carboxypeptidase A3 and MMP-9 are also present in the granules (Baram et al., 2001).

Activated MCs can also initiate the *de novo* synthesis of several lipid-derived molecules. Of significance are the arachidonic acid products, which have well-established inflammatory activities. One of the main lipoxygenase products is leukotriene C4 (LTC_4) (Dahinden et al., 1985, Sjostrom et al., 2002). The cyclooxygenase products of MCs include prostaglandin D2 and E2 (PGD_2 and PGE_2) (Metcalfe, 2008).

Class	Potential function
GRANULE-ASSOCIATED	
Histamine and serotonin	Alteration of the vascular permeability
Heparin and/or chondroitin sulphate proteoglycans	Enhancement of chemokine/cytokine function and angiogenesis
Tryptase. Chymase and other proteases	Tissue remodelling and recruitment of effector cells
TNF and VEGF	Recruitment of effector cells and increase of angiogenesis
LIPID-DERIVED	
LTC_4, PGD_2 and PGE_2	Recruitment effector cells, regulation of immune response, promotion of angiogenesis and oedema, bronchoconstriction
Platet-activating factor (PAF)	Activation of effector cells, enhancement of angiogenesis
CYTOKINES	
TNF, IL-1α, IL-1β, IL-6, IL-18, GM-CSF, LIF, INFA and INFβ	Inflammation
IL-10, TGFβ and bFGF	Regulation of inflammation and angiogenesis
CHEMOKINES	
CCL-2, CCL-3, CCL-4, CCL-5, CCL-11 and CCL-20	Recruitment of effector cells, including dendritic cells and regulation of immune responses
CXCL1, CXCL2, CXCL8, CXCL9, CXCL10 and CXCL11	Recruitment of effector cells and regulation of immune responses

Table 1-1 Predominant components of MC granules: preformed and de novo synthetized molecules. (Metcalfe, 2008).

1.3.4 MC activation

The activation of MCs is often referred to as degranulation, due to the physical release of mediators from MC granules by exocytosis into surrounding extracellular sites. The release may be induced by: 1. chemical substances, such as proteases; 2. endogenous mediators, tissue proteases or cationic proteins generated by other immune cells; 3. immune mechanisms which may be IgE dependent or independent, as illustrated in Figure 1-13, (Galli et al., 2011, Kawa, 2012). MC degranulation is preceded by an increase of extracellular Ca^{2+} influx, which is an essential step as the

inhibition of changes in the intracellular Ca^{2+} suppresses degranulation (Rice et al., 2013).

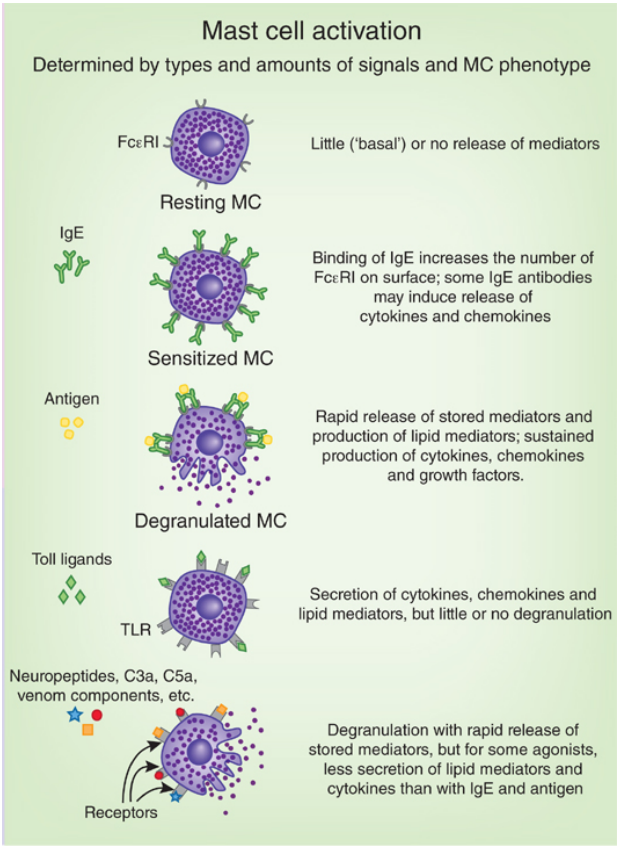

Figure 1-13 Patterns of functional MC activation may be IgE dependent or independent.
A) MC represented at its resting phase, where little or no release occurs. **B)** IgE association occurs during allergic reactions and interaction to parasites. IgE binds to the FcεRIα receptors on MC membrane and induces degranulation in the presence of an antigen **(C)**. **D-E)** Degranulation can be induced by signals not dependent on IgE, like neuropeptides such substance P, NGF and histamine, or C3a C5a of the complement system or toll-ligands. Image adapted from Galli et al. (2011).

Activation/degranulation occurs in two waves (Figure 1-14), first there is a rapid response that begins within seconds to minutes following stimulation, which is characterised by the release of the pre-formed mediators and the synthesis of lipid-derived eicosinoids which is transcription independent. In the second phase of activation, MCs initiate *de novo* synthesis of mediators, including a large number of cytokines, that are transcribed and translated in response to activation (Figure 1-14) (Abraham and St. John, 2010). MCs are known to be long lived cells (Padawer, 1974, Kiernan, 1979), and once degranulation is complete their granules are replenished and, the MCs re-enter their resting phase (Figure 1-14).

There is growing evidence that MCs have the capability to respond and degranulate in response to several types of stimuli, not only after IgE-antigen crosslinking. Various inflammatory products can induce MC activation, and these

include complement factors, toll-like receptor ligands (TRLs), cytokines, hormones and neuropeptides (Yu et al., 2016).

Maximal release happens within a few minutes while recycling of the membrane and granule replenishment occurs within the scale of a few hours to days.(Blank et al., 2014, Balseiro-Gomez et al., 2016). The mechanisms of granule retrieval, such as endocytic processes which induce retention of the intragranular matrix after degranulation, guarantee the maintenance of granule shape and proteoglycan matrix. They also ensure rapid granule recycling which maintains the secretory response during repeated stimulation.

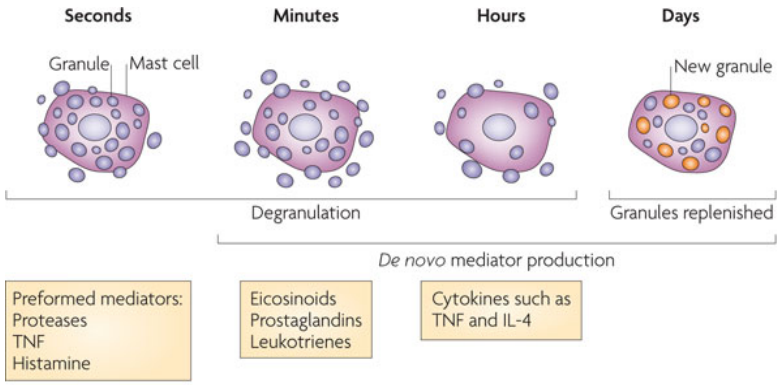

Figure 1-14 Time frame of MC degranulation and granule replenishment after stimulation.
MCs release pre-formed mediators within seconds after stimulation, within minutes the release of lipid-derived molecules occurs (without transcription and protein translation). A second wave of degranulation consists with the release of *de novo* synthetized molecules, which require translation and transcription, and therefore this happens within hours after MC activation. Granules are replenished within days after activation. Image adapted from Abraham and St John (2010).

1.3.4.1 Tryptase and histamine receptors

MCs express receptors that enable them to modulate the immune response in both an activating and inhibiting fashion. Examples include the protease-activated receptor 2 (PAR-2), which can be activated by tryptase and the histamine receptor 1 (HR1), which is activated by histamine. Both PAR-2 and HR1 belong to the G protein-coupled receptor family (GPCRs) which regulate availability of functions via downstream mediators including Ca^{2+} (for ion channels), cAMP and cGMP (for adenylyl and guanlyl cyclases), inositol-1, 4,5-triphosphate (IP3), diacyl glycerol (DAG) and arachidonic acid (for phospholipases) (Tuteja, 2009). In the case of PAR-2 and HR1 following their activation there is an increase in inositol triphosphate which leads to an increase of intracellular Ca^{2+} (Ossovskaya and Bunnett, 2004, Hill, 1990).

PAR-2 and HR1 other than being expressed by MCs, are also present in a wide variety of other cell types, including fibroblasts, endothelial cells, smooth muscle cells, immune cells and sensory neurons (Coughlin, 2000, Parsons and Ganellin, 2006).

PAR-2 receptor is uniquely activated by irreversible proteolysis. Unlike other GPCRs, the endogenous PAR ligands are resident in the extracellular matrix, near the N-terminus of the receptor (Figure 1-15). The cleavage of the receptor in the N-terminal domain by tryptase reveals the tethered ligands, which after being exposed, bind the second extracellular loops of the receptor structure (Soh et al., 2010, Zhao et al., 2014) initiating the signalling cascade.

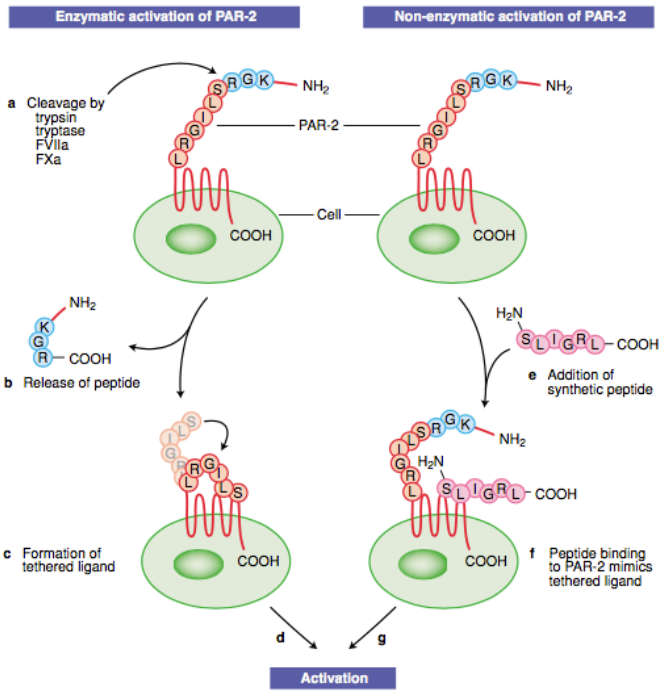

Figure 1-15 Two possible activation mechanisms of PAR-2 receptor: enzymatic and non-enzymatic way. **A)** Serine proteases such as tryptase can enzymatically cleave the N-terminal peptide of PAR-2, **B)** which induce the release a peptidic chain, and new N-terminal end formation **C)**, which can bind to the second transmembrane loop of the receptor. **D)** The formation of tethered ligand induce an intracellular G-protein-coupled pathway that results in intracellular Ca^{2+} increase and activation of protein kinase C. The non-enzymatic was involves a synthetic SLIGRL peptide **(E-F)** directly binds to the body of PAR-2 without the cleavage of the N-terminal peptide, therefore **G)** it mimics the effect of the PAR-2-activating proteases. (Kawabata, 2002)

Antagonism to PAR-2 and HR1 receptors has been shown to block MC activation (Levi-Schaffer and Eliashar, 2009), reduce tissue oedema and pain perception (Mobarakeh et al., 2000, Ferrell et al., 2003, Sevigny et al., 2011, Poole et al., 2013).

1.3.5 Tools for studying MCs

1.3.5.1 MC cell lines and MC primary cells

In 1988, the first human MC line was established from the peripheral blood of a patient with MC leukaemia, and named HMC-1 (Butterfield et al., 1988). HMC-1

cells share features with immature MCs, and they are independent of SCF for their growth, due to a point mutation in the CD117 (c-kit) receptor, which results in constitutive phosphorylation and activation of the receptor without the ligand. HMC-1 cells lack the well-formed granules seen in mature MCs, and not all the classic MC components are present e.g. chymase is almost absent. The proteoglycan structure of the few granules that are present is similar to that of primary MCs, and contain both heparin and chondroitin sulphate. Furthermore, HMC-1 cells have other critical limitations, for example they lack a functional IgE receptor (Nilsson et al., 1994a).

The development of other MC lines, such as LAD2, which was prepared from bone marrow aspirates of a patient with MC sarcoma/leukaemia, have improved the range of *in vitro* tools available to study MCs. LAD2 cells have numerous granules, degranulate in response to IgE-mediated activation, and are SCF dependent for their growth and survival and therefore share more features than HMC-1 with mature MCs (Kirshenbaum et al., 2003).

MCs have also been cultured from foetal liver, umbilical cord blood and peripheral blood (Metcalfe, 2008). Unfortunately, the micro-environmental conditions that influence full MC maturation *in vivo* are very difficult to repeat *in vitro*. Thus the cultured MCs that have been studied to date are imperfect representatives of mature MCs (Moon et al., 2010).

Although it is possible to isolate MCs from tissues that might provide important tissue specific information, this method also has associated limitations. Rodent peritoneal MCs (PMCs) can be easily collected in a relatively high number (Karimi et al., 1999), however, the human peritoneal cavity has few MCs and extrapolation of results from rodent PMCs to human MCs requires caution (Bischoff, 2007). Further limitations to the usage of peripheral tissue-derived MCs include: the labour-intensive nature of the isolation protocols; change of tissue-resident MC phenotype; and low yields of MCs. To date there have been reports that human MCs have been isolated from skin, intestine, lung and uterus (Massey et al., 1991, Gibbs and Ennis, 2001, Kulka and Metcalfe, 2001a, Sellge and Bischoff, 2006). Unfortunately, the effects of the isolation and enrichment procedures on MC functions has not yet been evaluated.

1.3.5.2 Animal models of MC deficiency

The *in vivo* relevance of *in vitro* observations have been tested using MC-deficient animal models, as this allows the study of MC function in complex *in vivo* settings that cannot be carried out in humans.

The use of MC-deficient mice and their reconstitution with bone marrow derived MCs (BMMCs knock-in model) has been a powerful tool to study MC function *in vivo*. For example, the importance of MCs in pregnancy establishment and labour initiation has been demonstrated using the knock-in system in the $Kit^{W\text{-}sh/W\text{-}sh}$ "sash" animals (Menzies et al., 2012, Woidacki et al., 2013b).

Animals with spontaneous loss-of-function mutations at both alleles of the dominant *white spotting* (*W*) locus, for example c-*kit* gene, present an evident impairment in the c-*kit* tyrosine kinase-dependent signalling, which results into altered mast cell development and survival (Grimbaldeston et al., 2005).

However, the interpretation of results obtained using MC-deficient animals should be treated with caution because they present with profound phenotypic abnormalities in both the hematopoietic and immune systems (Nocka et al., 1989).

1.4 Biological effects of MC granule components

MCs are implicated in the pathology of diverse conditions such as allergy, asthma, rheumatoid arthritis, interstitial cystitis, and inflammatory bowel syndrome (Theoharides et al., 2012). The mediators released by MCs can independently, and in synergy of other immune cell type cytokines (e.g. macrophages and T-cells derived), orchestrate a complex immune response.

The key events of MC degranulation and their putative impacts are summarized in Figure 1-16.

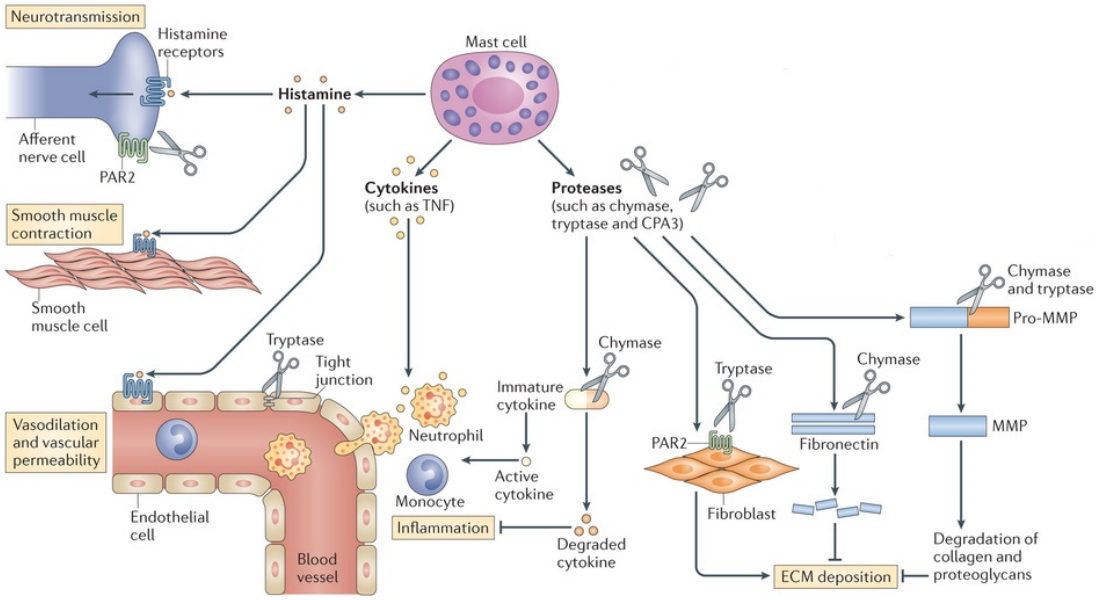

Figure 1-16 An overview of the possible biological effects of MCs.
Image adapted from Wernersson and Pejler (2014).

In brief, histamine released by MCs exerts many effects following binding to receptors such as HR1 including the stimulation of afferent sensory nerves (Kim et al., 2011), induction of smooth muscle contraction (Suzuki and Kou, 1983), increases in vascular permeability and vasodilation (Sorbo et al., 1994, Lu et al., 2013).

It has been demonstrated that MCs can actually store preformed cytokines within secretory granules, initially reported as tumour necrosis factor a (TNFα), which contributes significantly to the pro-inflammatory response (Wernersson and Pejler, 2014). MCs also produce and secrete a large number of other cytokines, growth factors and chemokines (Galli et al., 2005). These factors are mostly released as a result of do novo synthesis as opposed to preformed pools (Lundequist and Pejler, 2011). However, there are a number of factors/cytokines that may actually be stored in MC granules including vascular endothelial growth factor (VEGF) (Grutzkau et al., 1998), transforming growth factor-b (TGFβ) (Lindstedt et al., 2001).

The MC-specific proteases which are abundant in MC granules, have a significant impact both through the activation of protease-activated receptor 2 (PAR-

2) and other structural components. For example, the cleavage of PAR-2 by tryptase results in the activation of afferent nerves, leading to an increase in pain perception (Hoogerwerf et al., 2001), and in activation of fibroblasts to stimulate consequent extracellular matrix (ECM) deposition and fibrosis (Akers et al., 2000). Moreover, chymase can also promote tissue remodelling either directly by degrading the ECM fibronectin (Okumura et al., 2004), or indirectly by activation of the pro-MMPs into proteolytic MMPs (Tchougounova et al., 2005, Magarinos et al., 2013)

1.4.1 MCs in tissue remodelling and fibrosis

Direct action of MC proteases on ECM components includes the degradation of fibronectin by chymase (Okumura et al., 2004). As an important example of an indirect role, chymase has been reported to have pro-collagenase activity (Saarinen et al., 1994) and is essential for transforming the pro-enzymes for matrix metallo-proteinase 9 (pro-MMP-9) and pro-MMP-2 into active enzymes (Tchougounova et al., 2005). Similarly, tryptase has been demonstrated to activate MMP-3 and MMP-13 (Magarinos et al., 2013).

There is also evidence that MC proteases can stimulate pro-fibrotic activity by inducing fibroblast proliferation via PAR-2 receptor cleavage (Akers et al., 2000, Duchesne et al., 2011) and that chymase can activate transforming growth factor-β (TGFβ) (Lindstedt et al., 2001), therefore facilitating pro-fibrotic activity. It is also likely that protease-mediated effects on the ECM could further support angiogenesis, as this could be augmented by the release of VEGF from preformed stores in granules (Grützkau et al., 1998).

1.4.2 MCs and angiogenesis

MC are often associated with blood vessels and their numbers increase during angiogenesis-dependent events like ovulation and wound healing (Norrby, 2002).

The stimulatory action of MC mediators can affect different stages of angiogenesis such as degradation of the ECM, migration and proliferation of endothelial cells, formation and distribution of new vessels, synthesis of ECM, and pericyte mobilization (de Souza Junior et al., 2015).

Therefore, the biological consequence of MC activation on the vasculature has been extensively studied. It is known that the micro-vascular responds to granule-derived mediators such as histamine, lipid-derived PGD_2, LTB_4, LTC_4, and cytokines like VEGF and PAF (platelet activation factor), all present or sequentially synthetized and stored in MC granules. All these agents are able to augment micro-vasculature permeability, which has pro-angiogenic effects (Dvorak et al., 1999). Moreover, PAF is also a powerful chemotactic agent for eosinophils and neutrophils and causes platelet aggregation (Norrby, 2002).

Furthermore, it has been demonstrated that tryptase and chymase can also exert angiogenic effects. For example, it has been shown that tryptase acts as a mitogen on micro-vascular endothelial cells enhancing capillary growth, an effect that can be suppressed by tryptase inhibitors (Caughey et al., 1993, Blair et al., 1997). For chymase, it has been shown that its angiogenic action is indirectly related to the role of angiotensin II (Ang II) (Muramatsu et al., 2000b). Chymase, like the angiotensin converting enzyme (ACE), can cleave angiotensin I into angiotensin II isoform, which has been reported to be a potent inductor of neovascularization via VEGF (Muramatsu et al., 2000a). Notably, chymase is more efficient that ACE in producing this conversion. It has been also demonstrated that Ang II can increase blood flow by ischemia-induced angiogenesis (Sasaki et al., 2002) highlighting the diverse impact of chymase on the vasculature.

Interestingly, Ang II has been shown to positively influence the proliferation of endothelial cells and vascular smooth muscle cells *in vitro* (Tamarat et al., 2002). Moreover, Ang II is a potent stimulus for VEGF-induced proliferation and tube formation in retinal microvascular endothelial cells, through the induction of the VEGF receptor (Otani et al., 1998).

1.4.3 MCs and nerve interaction, pain perception

MCs are critical cellular regulators of physiological and pathological pain pathways. Pain can be classified as either a protective or a maladaptive process. Protective pain includes nociceptive pain which triggers nerve signals to ensure rapid removal from an intense stimulus (Figure 1-17-A). Protective pain also includes the inflammatory-induced pain that is associated with tissue damage (Figure 1-17-B), which promotes immune cell influx and hypersensitivity until the tissue has been repaired (Woolf, 2010). Pathological pain, which is a result of a maladaptive pain, is of a chronic nature, it doesn't resolve rapidly and is often associated with long term symptoms (Figure 1-17-C). The intimate interaction between sensory nerves and the immune system plays a key role in pain processing, and in the fine balance between protective and pathological pain (Ren and Dubner, 2010).

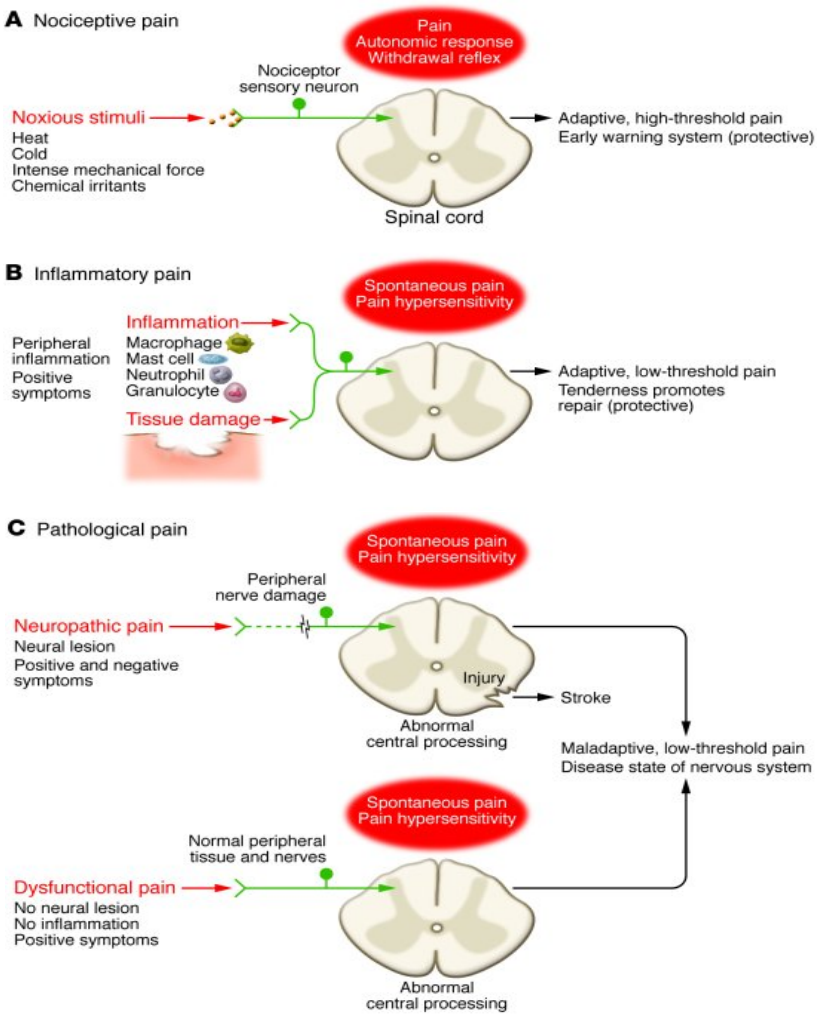

Figure 1-17 Three classes of pain.
A) Nociceptive pain responds to a detection of potential tissue-damaging stimuli and is considered a protective type of pain, **B)** Inflammatory pain occurs after tissue damage, with the influx of several immune cells. Hypersensity may occur until full resolution. It is a protective type of pain, **C)** Pathological pain occurs in the case of damage to the nervous system (neuropathic type, top right) or in response of the abnormal fuction (dysfunctional type, bottom right) (Woolf, 2010).

MCs are frequently associated with nociceptive neurons and may reside in close proximity to unmyelinated nerve fibres, such as nociceptive C-fibres (Forsythe and Bienenstock, 2012). MCs can release an array of mediators with neuro-inflammatory proprieties including histamine, serotonin, IL-1β, TNFα and IL-6 which have been demonstrated to induce hyperalgesia in either an independent manner or in association with chemical or allergen stimulation (Cunha et al., 1992, Lavich et al., 2006, Verri et al., 2006). It has been postulated that there is a positive feedback loop between nociceptive neurons and MCs during neuro-inflammation (Rosa and Fantozzi, 2013), where neuronal fibres activate proximal MCs by releasing neuromodulators and subsequently MCs release algogenic molecules that then amplify vasodilation and sensitization of nociceptors (Figure 1-18) (Koda and Mizumura, 2002). This relationship between sensory nerves and MCs has also been supported by the use of MC stabilizers which were shown to inhibit nociception (Le Filliatre et al., 2001).

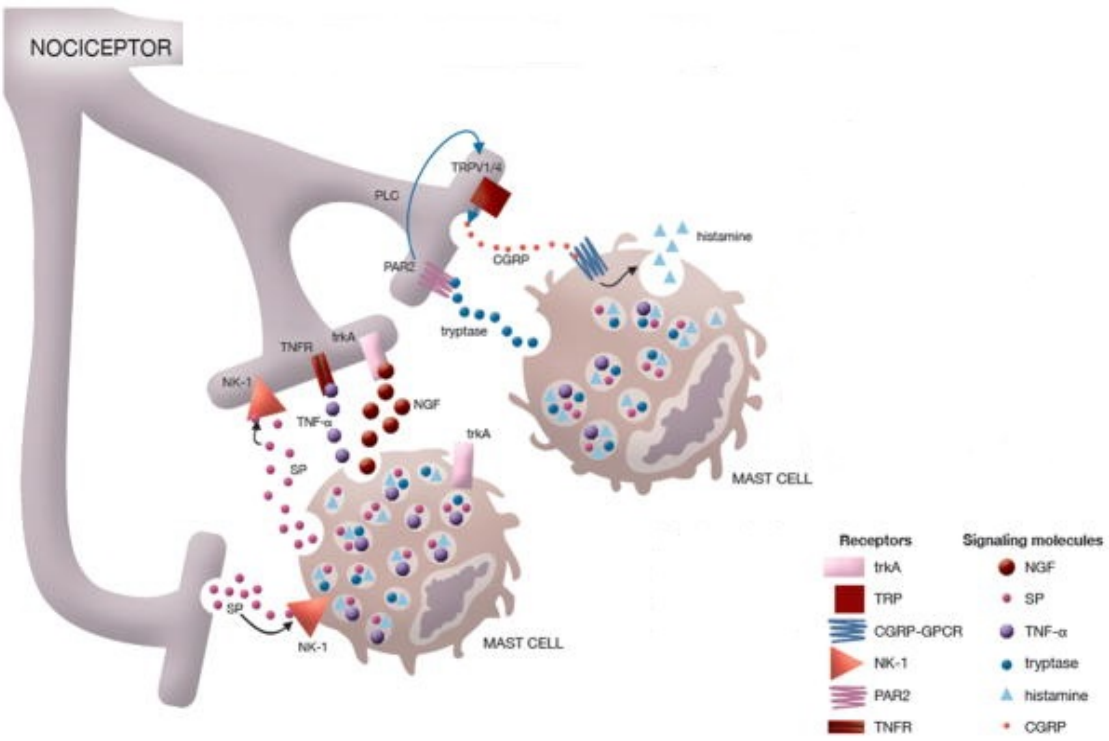

Figure 1-18 Potential interactions between sensory nerves and MCs.
Both neurons and MCs have the potential to produce and then secrete an array of neuro-inflammatory mediators, such as NGF, substance P (SP), which can bind their receptors (trkA and NK-1, respectively). NGF and SP can also take part in nociceptive signalling. MC specific tryptase may bind PAR-2 receptor on neurons and initiate inflammatory cascades, which include the release of CGRP (calcitonin gene related peptide). CGRP receptors are expressed on MCs, and their stimulation leads to histamine release from the granules. Image adapted from Chatterjea and Martinov (2015).

Another key signalling molecule between nerves and MCs is substance P. This factor can be released by both neurons and MCs and leads to a sequential production of histamine and prostaglandins (Janiszewski et al., 1994, Theoharides et al., 2012). Histamine is also released from MCs and directly excites nociceptors. Several studies have demonstrated that histamine H1 receptors have a critical role in physiological and pathological pain perception (Foreman et al., 1983, Mobarakeh et al., 2000).

The release of the MC-specific protease tryptase can also cause hyperalgesia upon binding to PAR-2 expressed on the nociceptors (Amadesi et al., 2006). PAR-2-mediated hyperalgesia has been shown to depend on TRPV4, a member of the transient receptor potential cation channel subfamily V, in a model of colorectal distension (Sipe et al., 2008). Moreover, tryptase-induced PAR-2 activation can also trigger the release of calcitonin gene-related peptide (CGRP) by neurons, which in turn stimulates histamine from MCs (Ottosson and Edvinsson, 1997). MCs in women health

Substantial differences in the inflammatory responses of women compared with those of men have been identified (Grossman, 1989, Schuurs and Verheul, 1990). It has also been reported that the incidence and the severity of immune disorders are generally higher in women than in men and included in this observation were MC-related painful conditions such as asthma, irritable bowel syndrome (IBS) and interstitial cystitis (IC).

For example, the prevalence of asthma is higher in boys than girls during puberty (Bonds and Midoro-Horiuti, 2013) but switches during adulthood so that women then have a greater incidence of asthma exacerbations which are also much more severe than in men (Leynaert et al., 2012). Highlighting a link to steroid hormones are reports that asthma worsens in women during the perimenstrual phase of their cycle, and that an alleviation of asthmatic attacks occurs after menopause (Romieu et al., 2010).

Adult premenopausal women suffering from IBS present with a higher prevalence of symptoms and a lower quality of life compared to men, and have worse symptoms perimenstrually (Canavan et al., 2014, Heitkemper and Chang, 2009). It is reported that the total count of immune cells is increased in intestinal tissue biopsies from women compared to men (Barbara et al., 2004, Cremon et al., 2009).

Similar characteristics are reported for women with IC, with 90% of IC patients being female, and they report a worsening of IC pain symptoms during the postovulatory phase (Bjorling, 2001).

An association between the actions of female sex hormones, oestrogen and progesterone, and symptoms of MC-associated disorders has been extensively studied.

1.4.4 MC oestrogen receptor expression

The first evidence of the expression of oestrogen receptor (ER) in human MCs was reported by Pang et al. (1995). He and his colleagues reported that MCs appeared both numerous and immunopositive for ER (ER isoform not specified) in bladder biopsies from patients with interstitial cystitis (IC). This finding was the first to support the hypothesis that oestrogen could have direct receptor mediated effects in a MC-related condition.

Following this observation, other scientists have evaluated the presence of ER positive MCs in other tissues considered to be MC targets. These include nasal polyps (Zhao et al., 2001), a condition that often coexists with asthma, and the examination of aortic wall, as site of vascular MCs (Nicovani and Rudolph, 2002). Once again, MCs were found to be immunopositive for ERs in both of these tissues.

Once the presence of ER in the nuclei of mature MCs was confirmed, it was shown that MCs can respond to oestrogen (E_2) and sequentially degranulate and release the granule-derived compounds, including cytokines, such as TNFα and IL-6 (Theoharides et al., 1993, Kim et al., 2001). The activating action of oestrogen has also been implicated in studies on environmental compounds with oestrogenic activities (Narita et al., 2006), which have been implicated in the rise in prevalence and morbidity associated with allergic disease, including asthma, in industrial countries (Burr et al., 2006).

The mechanism by which E_2 induces MC activation was defined as being dependent on ERα not ERβ, using bone marrow-derived MCs from ERα knockout animals and the HMC-1 line (Narita et al., 2006, Zaitsu et al., 2007, Jensen et al., 2010).

1.4.5 MC progesterone receptor expression

Fewer studies have investigated the expression of progesterone receptor (PR) in MCs. A small percentage of MCs in IC biopsies were reported to be immunopositive for PR, which was low when compared to the 100% that were reported to be ER-positive (Theoharides et al., 1993, Pang et al., 1995, Letourneau et al., 1996), suggesting some heterogeneity may exist between MCs in the bladder. Zhao et al. (2001), investigated PR expression together with ER expression in MCs in the upper airways, reporting MCs were immunopositive for both. Studies employing HMC-1 cells have also demonstrated the nuclear expression of PR (Letourneau et al., 1996, Jensen et al., 2010).

Interestingly, there is contradictory evidence on the impact of progesterone on MC activation. Anti-inflammatory effects were illustrated by Vasiadi et al. (2006), where progesterone was shown to inhibit histamine release after MC stimulation with substance P and c48/80, both MC secretogue compounds. In contrast, pro-inflammatory effects were demonstrated by Jensen et al. (2010), in combination with oestradiol.

1.5 Endometriosis: an incurable gynaecological condition

Endometriosis is a benign, oestrogen-dependent, chronic gynaecological disorder associated with pelvic pain and infertility (Giudice and Kao, 2004). It is defined as the presence of endometrial tissue outside (ectopic lesion) of the normal uterine location (eutopic endometrium). The majority of lesions are found on the pelvic peritoneum, but also may be detected on the ovaries (endometriomas) and in the rectovaginal septum and in the pouch of Douglas (as illustrated in Figure 1-19). Classification of endometriosis is based on criterion established by the American Society for Reproductive Medicine (ASRM) ranging from mild endometriosis (grade I) to severe (IV) (Canis et al., 1997).

The prevalence of pelvic endometriosis is estimated to be approximately 6–10% of the total population of women of reproductive age (Eskenazi and Warner, 1997). In

women suffering from pain, infertility, or both, the frequency of endometriosis is up to 35–50% (Cramer and Missmer, 2002, Janssen et al., 2013).

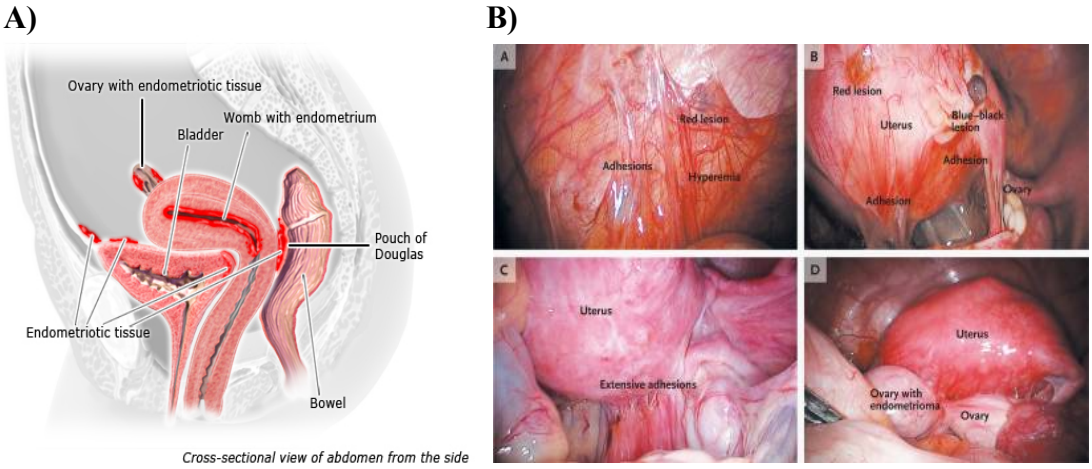

Figure 1-19 Representation of possible sites of endometriotic lesions and their morphology during laparoscopic investigation.
A) The most common endometriotic lesion sites are on the pelvic peritoneum (bottom left), in the pouch of Douglas and on the ovaries, image retrieved from the Institute for Quality and Efficiency in Healthcare (IQWiG) **B-A)** Endometriotic lesion (red type), adhesions, and hyperemia in the peritoneum. **B-B)** Two types of peritoneal lesions, including red and blue–black lesions and adhesions. **B-C)** General adhesions distorting the normal pelvic anatomy. **B-D)** Ovarian endometriotic lesion (endometrioma), (Giudice, 2010).

The most widely accepted theory explaining the establishment of endometriotic lesions was proposed by Sampson (1927) as the theory of "retrograde menstruation". He proposed that the shed endometrium which is fluxed back via the Fallopian tubes into the peritoneal cavity during menstruation becomes implanted in extra uterine sites. Other theories also exist and a full understanding of the aetiology and pathogenesis remains uncertain (Young et al., 2013).

Endometriosis is frequently under-diagnosed and menstruation occurs as retrograde in up to 90% of healthy women (Halme et al., 1984) and this, together with its complex presentation is believed to account the average of 6.7 years from the onset of symptoms before a definitive diagnosis following surgical investigation. Endometriosis is a debilitating condition which can dramatically affect the patient's quality of life (Oehmke et al., 2009, Facchin et al., 2015). It is further considered to have a significant societal impact; a European multi-centre study has estimated that the direct cost per endometriosis patient is up to 3113€ per annum, similar to other chronic diseases such as diabetes (2858€/year) (Simoens et al., 2012). In addition, the indirect costs of endometriosis-associated symptoms on loss of productivity are twice

as large as the direct health care costs mentioned above, being comparable with those associated with rheumatoid arthritis.

No definite cure exists for endometriosis, and the current treatment options include either the surgical removal of endometriotic lesions by laparoscopy, and medical therapies that aim to the relieve pain and ameliorate infertility, or both, detailed in section 1.5.5 (Giudice and Kao, 2004).

1.5.1 Evidence that lesions have an altered hormonal environment

Steroid hormones may have an effect on the proliferation of endometrial cells, their capacity to attach onto the peritoneal mesothelium and their evasion of immune mediated clearance. The hypothesis that endometriosis is an oestrogen dependent condition has supported by a number of studies (Kitawaki et al., 2002). The striking finding in endometriotic lesions compared to the eutopic endometrium is the elevated expression of the aromatase enzyme and reduced expression of 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2 (Zeitoun et al., 1998, Zeitoun and Bulun, 1999). The consequence of this enzymatic disparity is an increase in the local bioavailability of E_2. The available E_2 may also stimulate the production of eicosanoids within the lesions including prostaglandin E_2 that can further stimulate aromatase enzyme activity (Noble et al., 1997). These pioneering findings have been recently complemented by use of chromatography mass spectrometry and evaluation of mRNA concentrations highlighting the importance of local "intracrine" metabolism of oestrogens in detecting the steroid environment of the lesions (Huhtinen et al., 2012). Furthermore, studies have highlighted that oestrogen may influence mature MC behaviour for example E2 induces recruitment/movement and triggers degranulation *in vitro* (Jensen et al., 2010). Therefore, locally produced E_2 in endometriotic lesions may act as an enhancer of MC activities in the peritoneal cavity of women suffering from endometriosis, contributing to aberrant inflammatory processes.

There is also evidence that "progesterone resistance" is also important in the pathophysiology of endometriosis (Bulun et al., 2006). For example, endometriotic lesions display a significant reduction in progesterone receptor (PR) expression

compared to the eutopic endometrium, and a complete ablation of the isoform B of PR (Attia et al., 2000). Furthermore, the gene profiling of the eutopic endometrium has revealed a dysregulation of progesterone responsive genes during the luteal phase (Burney et al., 2007) of women with endometriosis. An imperfect proliferation to secretory phase transition of the endometrium may result into molecular implications that might support the survival and implantation of shed endometrium but this requires further investigation.

1.5.2 Peritoneal endometriosis

1.5.2.1 Physiology of the peritoneum

The human peritoneum is the largest serous membrane with the body (approximately 1.8 m^2) (Michailova, 2006). It consists of a layer of loose connective tissue covered by the mesothelium over the basal lamina (Figure 1-20). The

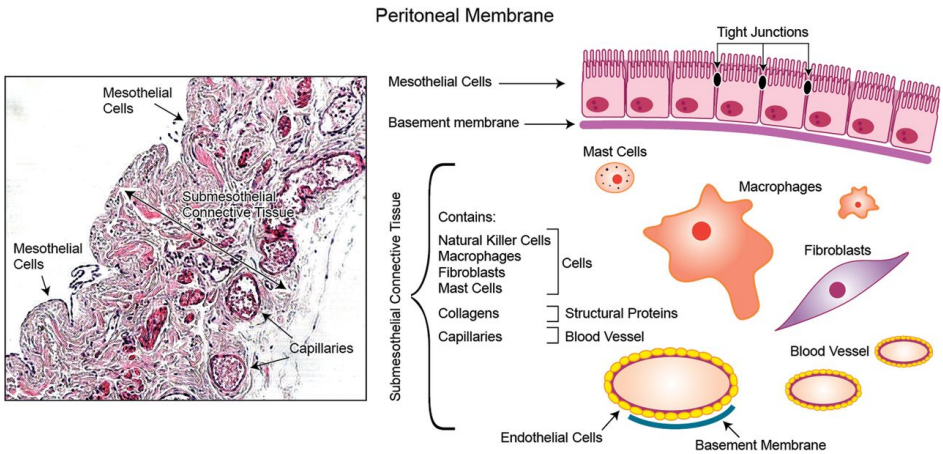

Figure 1-20 Histological appearance of the human peritoneum and schematic structure of peritoneal cellular compartments.
(Young et al., 2013)

mesothelium is an epithelial-like monolayer that covers the surface of the peritoneal membrane. The mesothelium protects organs from movement friction and a small volume of peritoneal fluid is present to further enhance this frictionless environment (Blackburn and Stanton, 2014).

The mesothelial cells are responsible for the physiological homeostasis of the peritoneal cavity. The peritoneum, sometimes referred to as the peritoneal "membrane", is also important for the transport of solutes and fluids to blood vessels, and participates in host defence. The tight junctions between the mesothelial cells

control the transport of ions, water and other solutes ensuring the unique microenvironment of the peritoneal fluid is maintained.

The peritoneal membrane can also contribute to host defence by secreting inflammatory cytokines which subsequently recruit immune cells such as neutrophils and macrophages (Jonjic et al., 1992, Lanfrancone et al., 1992). In the sub-mesothelial layer within the collagen mesh, there are networks of capillaries surrounded by a variety of cell types including fibroblasts, macrophages and mast cells, which can also interact with the underlying adipose tissue in a paracrine manner (Shimotsuma et al., 1993, Michailova, 2006). The peritoneum includes afferent nerves, many of which appear to be of the sensory type (Bentley et al., 1981, Tanaka et al., 2002).

1.5.2.2 Peritoneal fluid

The peritoneal fluid derives from plasma transudate and ovarian exudate in women (Koninckx et al., 1980) and its volume varies throughout the menstrual cycle, typically being between 5 and 20 ml with the largest volume during the secretory phase (Oral et al., 1996a). In women peritoneal fluid is considered to be a sex steroid reservoir (Kim-Bjorklund et al., 1991, D'Hooghe et al., 1995). The fluid contains a variety of immune cell types including natural killer cells (NKs), macrophages and mast cells (Hill et al., 1988, Weil et al., 1997, Xu et al., 2002). The physiological characteristics and any pathological changes in the peritoneal fluid are important contributors to the health and/or disease of the peritoneum (Young et al., 2013).

1.5.2.3 Lesion formation and cell invasion

Although, endometriosis is considered a benign disorder, the processes of attachment and invasion of the endometrial cells shares some features with those of malignant cells.

Once attached, ectopic endometrial cells must invade the peritoneal layer to form endometriotic lesions which are sustained by development of a blood supply. It is not clear yet whether the ectopic endometrium requires the mesothelium to be damaged in order to establish a lesion or if it can adhere directly to an intact mesothelial cell layer (Young et al., 2013).

Notably, studies using endometrial and peritoneal biopsies from patients with and without endometriosis revealed there was an increase in the invasiveness of the endometrial stromal cells in the endometriosis group (Shi et al., 2011). This endometrial cell invasion may be facilitated by MMPs, which are able to digest and remodel the ECM. MMPs are present in the normal functional endometrium at the time of menses and it has been shown that they are expressed in endometriotic lesions and also in the peritoneal fluid (Gilabert-Estelles et al., 2007, Itoh et al., 2012, Chen et al., 2013). Studies using primary mesothelial cells from women with endometriosis have shown that they have an altered metabolic profile (Young et al., 2014), which may also encourage formation of lesions. The role of the mesothelium in establishment of lesions is an active area of research with a number of studies identifying altered expression of regulatory molecules including TGF-β (Young et al., 2015, Young et al., 2016).

1.5.3 Endometriosis associated inflammation

Endometriosis is considered to be a neuroinflammatory disorder and cells of the immune system are believed to play an important role in the pathogenesis of the disorder (Lebovic et al., 2001) with inadequate immune cell surveillance within the peritoneum proposed as a factor in its aetiology. For example, peritoneal NKs from women suffering from endometriosis show a reduced cytotoxicity when compared with those from healthy controls which may allow ectopic endometrial cells to avoid detection (Oosterlynck et al., 1991, Quaranta et al., 2006). These findings complement those showing that a decrease in peritoneal NK cytotoxicity was correlated with a more severe endometriosis stage (Funamizu et al., 2014).

Macrophages are the most abundant immune cell type in the peritoneal fluid and many studies have investigated whether they may play a role in the pathogenesis of endometriosis (Ho et al., 1997, Bacci et al., 2009, Capobianco and Rovere-Querini, 2013). Peritoneal macrophages have been recorded as more numerous in endometriosis patients, and they appear to be activated (Oral et al., 1996a, Tran et al., 2009). High concentrations of macrophages are also reported to be associated with the endometriotic lesions themselves, especially with the highly inflamed “red” endometriotic lesions (Zeller et al., 1987, Khan et al., 2004). These findings were also

recapitulated in a mouse model of endometriosis using transgenic mice in which cells of the monocytes lineage were labelled with GFP. GFP positive cells were readily detected in the induced endometriotic lesions and shown to be derived from endometrium or peritoneum (Greaves et al., 2014b).

Increasing evidence has also supported the possible role of mast cells (MCs) in the altered inflammatory environment of endometriosis. High numbers of degranulating MCs have been observed in association with endometriotic lesions (Matsuzaki et al., 1998b, Sugamata et al., 2005). Furthermore, stem cell factor (SCF), which is important for MC number, has been reported to be elevated in the peritoneal fluid of endometriosis patients (Osuga et al., 2000).

An increase in the number of immune cells might lead to sustained inflammation with an increasing production of inflammatory cytokines that would not only affect the endometriotic lesions but also the surrounding area. Indeed, the peritoneal fluid from women affected by endometriosis showed elevated concentrations of cytokines, growth and angiogenic factors, (Gazvani and Templeton, 2002), which may be produced directly by the lesions, or released by immune cells. After endometriotic lesions become established they are thought to secrete pro-inflammatory molecules into the peritoneal environment. It has been proposed that cytokines including IL-1, IL-8, TNFα and interferon γ present in the peritoneal fluid may act as chemotactic agents to increase the recruitment of macrophages and T lymphocytes to the peritoneum (Giudice and Kao, 2004) but further studies are required to confirm this.

It has been hypothesised that endometriosis shares features with autoimmune disorders, with reports of an increase in polyclonal B-cell activity, alterations in the function and number of B and T cells, and evidence of familial inheritance (Badawy et al., 1987, Lebovic et al., 2001, Nothnick, 2001). Additionally, high concentrations of IgG, IgA, and IgM autoantibodies have been revealed in serum and peritoneal fluid from patients with endometriosis (Badawy et al., 1987, Gleicher et al., 1987). Studies in patients have also revealed that there is a tendency for autoimmune diseases such as lupus erythematous, rheumatoid arthritis; and atopic conditions like asthma and

eczema to overrepresented in women with endometriosis (Sinaii et al., 2002, Caserta et al., 2016). Translation of these findings for patient benefit is awaited.

1.5.4 Endometriosis-associated pain and neuro-inflammation

Many of the most distressing symptoms among women suffering from endometriosis are pain-related, and these include dysmenorrhea (pain at menstruation), dyspareunia (pain during sexual intercourse) and chronic pelvic pain (CPP) (Kobayashi et al., 2014). Pain is often more severe during and before menstruation with a recent review proposing the shed menstrual tissue and the lesions themselves as the "trigger" for inflammatory pain in endometriosis (Laux-Biehlmann et al., 2015).

It has been postulated that endometriosis-associated CPP may be generated through a number of different pathways including one suggestion that endometriotic lesion establishment could provoke a compression or infiltration of nerves (Anaf et al., 2000). Alternatively it has been postulated that increasing concentrations of nerve growth factor (NGF), together with other neuro-inflammatory molecules such as TNFα, found in the endometriotic lesion and surrounding tissue area results in hyperalgesia. Finally, the altered inflammatory microenvironment may also promote increased "neural sprouting" stimulating the growth of new neuronal afferents within the lesion and additional interaction with nervous system (Arnold et al., 2012). The cocktail of neuro-inflammatory mediators found in the lesions and peritoneum of women with endometriosis may also exacerbate the CPP (Cheng and Ji, 2008).

The nerve fibres detected in endometriotic lesions are a mixture of Aδ and C sensory, cholinergic and adrenergic nerve fibres which have often been found to colocalize (Tokushige et al., 2006). Interestingly, both mature nerve endings and newly grown nerve fibres have been detected in endometriotic lesions, with the latter being associated with immature blood vessels (Tulandi et al., 2001). This close association between nerves and blood vessels has led to the proposal that neuroangionesis should be considered both important to the aetiology of the disorder and also a therapeutic target (Asante and Taylor, 2011).

There is also cumulative evidence that these new sensory fibres innervating endometriotic implants release pro-inflammatory compounds which in turn support

the heightened inflammatory state in the lesion. Conversely, nerve fibres themselves may become sensitized through the pro-inflammatory environment (Stratton and Berkley, 2011).

The secretion of potent neuroactive molecules by the endometriotic lesions or nerves may also promote a pro-inflammatory environment by recruiting immune cells, such macrophages, and by activating tissue resident leukocytes, such as MCs (Horigome et al., 1994). It is notable that MCs have been reported in close association with nerves in both peritoneal endometriosis (Kempuraj et al., 2004) and deep infiltrating endometriosis (Anaf et al., 2006) (recto-vaginal infiltrative lesions that affect vital structures such as bowel, ureters, and bladder (Vercellini et al., 2004)). These findings have led to the suggestion that MCs play a role in pain perception in women affected by endometriosis (Kirchhoff et al., 2012).

1.5.5 Current treatments for endometriosis

Three types of treatment have been used to alleviate pain and regress the lesions in women with endometriosis: non-steroidal anti-inflammatory drugs (NSAID) and other analgesics, hormonal agents, and the surgical removal of the endometriotic lesions. The choice of treatment may depend on the severity of the disease (Brown et al., 2014).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used to relieve dysmenorrhea (pain at menstruation). One randomized, controlled trial presented no significant reduction in endometriosis-associated pain with the use of NSAIDs as compared with placebo, and furthermore no difference between different NSAID drugs (Allen et al., 2005).

Hormonal therapy aims to suppress ovarian function thereby reducing circulating oestrogens and is employed to reduce proliferation/growth of lesions and associated pain. Hormonal therapy includes progestins, androgens, GnRH agonists, and contraceptive steroids (combination of oestrogens and progestins) (Vercellini et al., 1997, Practice Committee of American Society for Reproductive Medicine, 2008, Abou-Setta et al., 2013).

Surgical approaches to relieve endometriosis-related pain are often used as first-line therapy or initiated after failed medical therapies (Duffy et al., 2014). Surgical procedures include excision, laser fulguration, or laser ablation of endometriotic implants on the peritoneum (Giudice, 2010).

While most patients have improvement in symptoms with medical or conservative surgical interventions, >50% will experience recurrence of pain after medical treatment is discontinued and within 5 years after surgery (Falcone and Lebovic, 2011). Moreover, long-term efficacy of medical treatment, both analgesic and hormonal, is frequently limited by cost and adverse side effects (Lindsay et al., 2015). Therefore, the development of new treatment options that may overcome these obstacles remains a lay focus of the academic and pharmaceutical communities (Rogers et al., 2016).

1.6 General conclusions and aims of the study

The human endometrium, located in the centre of the uterus is a dynamic tissue, which cyclically experiences extensive tissue remodelling driven by the fluctuations in ovarian-derived hormones, most notably oestrogens and progesterone originating by the follicles and corpus luteum respectively. The endometrium is a multi-cellular tissue, which comprises of stromal and epithelial compartments and is usually classified into functional and basal regions with the former shed at the time of menses. The endometrial stroma is predominately formed by fibroblasts, a well-developed vasculature and hosts a diverse leukocyte population.

MCs are long-lived tissue resident immune cells characterised by the presence of granules containing proteases. Their phenotype is determined by the surrounding tissue environment, and in humans they are generally classified as being MC_T or MC_{TC} subtypes, depending on their expression of the MC specific proteases, tryptase and chymase. Mast cells have been detected in the human uterus, both in the myometrium and in the endometrium, but little is known about their regulation or the impact of steroids on their differentiation status in this organ. Studies on non-reproductive tissues have reported an impact of sex steroid hormones on MC behaviour.

Recently MCs have been associated with the physiology and pathology of different pain pathways but their role in endometrial pathologies has not been investigated. Endometriosis is an oestrogen-dependent, neuroinflammatory, incurable, gynaecological disorder characterized by the presence of endometrial tissue outside the uterine cavity, mainly on the pelvic peritoneum.

Women affected by endometriosis can experience debilitating symptoms, including chronic pain. Endometriosis symptoms are thought to be the result of an excessive inflammatory environment within the pelvis. Aberrant inflammation is not only present in the endometriotic lesions but also in the eutopic endometrium of patients with endometriosis.

Although the aetiology of endometriosis is uncertain, reports of close proximity between activated MCs and sensory nerves in endometriotic lesions, has suggested the MCs may contribute to endometriosis-related chronic and neuropathic pain.

Since there is evidence of MCs in the human endometrium that can degranulate in response to sex steroids the overarching hypothesis is that MCs could be involved in the establishment and maintenance of endometriosis.In the current study had the following aims:

1. To determine the spatial and temporal location of uterine MCs and to explore their phenotype including expression of steroid receptors,

2. To investigate the presence and activation status of MCs in women with endometriosis and/or pain,

3. To explore the use of primary cells, well-established cell lines, and mice as models to study the impact of sex steroids on MC phenotype and influence of their behaviours.

Chapter 2 Materials and Methods

2.1 Human tissues biopsies

2.1.1 Tissue collection

Human tissue was obtained from women undergoing surgery for non-malignant gynaecological conditions or sterilisation procedures. All patients were within the 25-50 year age range, had regular menstrual cycles, between 25-35 days, and had not taken exogenous hormones within three months prior to surgery.

Local ethical committee approval was granted and written informed patient consent was obtained prior to tissue collection. Ethical approvals were held by Professor H.O.D. Critchley (Number LREC 10/S1402/59 and 16/ES/0007) and Professor A.W. Horne (Number 11/AL/0376).

Blood samples obtained at the time of surgery were used to evaluated peripheral sex steroid hormone levels by Dr Forbes Howie (Specialist Assay Service, Surf Facility, University of Edinburgh). Endometrial cycle stage was confirmed by examination of tissue sections by an expert pathologist, Professor A.R.W. Williams (NHS, Royal Infirmary, Edinburgh).

2.1.2 Tissue processing

Tissue samples were either placed into 4% neutral buffered formalin (NBF) for future tissue processing and paraffin embedding (Section 2.5.1) or into RNAlater (Ambion, ThermoScientific, Paisley, UK) for subsequent RNA extraction (Section 2.2.1).

2.2 RNA extraction

RNA extraction was performed using the RNeasy mini kit (Qiagen, UK) for tissue samples and cell lines and the PureLink RNA Micro kit for RNA isolation from primary cell culture (Section 0, Invitrogen, UK). After tissue lysis, samples were homogenized and centrifuged. Total messenger RNA (mRNA) was eluted in RNA-free water following the manufacturer's protocol. mRNA enrichment was ensured by appropriate tRNA and rRNA size exclusion during this extraction protocol.

Every RNA extraction was carried out in RNA free conditions using RNase zap

(ThermoFisher Scientific, UK), sterile, filtered tips and dedicated pipettes were used to reduce contamination and avoid RNA degradation.

2.2.1 RNA extraction from human tissue biopsies

RNA was obtained from frozen biopsies immersed in RNAlater® (Ambion, Life Technologies, UK). Tissue biopsies were weighed and 20mg of tissue were cut into smaller pieces with sterile scalpels, to avoid saturating the RNeasy columns. Selected tissue pieces were minced and 1ml of TriReagent (Sigma-Aldrich, UK) was added in a 2ml Eppendorf tube containing a stainless steel bead (5mm, Qiagen, UK) to ensure efficient tissue lysis. Samples were lysed using a TissueLyser (Qiagen, UK) at a frequency of 25Hz for 2x3 minutes, followed by a 5-minute incubation at room temperature (RT), centrifugation for 15 minutes at 14,000g at 4°C for removal of tissue debris. The lysate was transferred into a pre-centrifuged 2ml PhaseLock "Heavy Gel" tube (5Prime, UK) with 200µl of chloroform (Sigma Aldrich, UK), forming 2 phases: aqueous and phenolic phase. The two phases were mixed thoroughly for 15 seconds and incubated for 5 minutes at RT, ensuring formation of an emulsion and subsequent RNA/protein separation. The mixture was centrifuged at 14,000g for 15 minutes at 4°C to complete the RNA/protein separation. The RNA containing phase (aqueous fraction) was then pipetted off into a new tube.

Samples were desalted and concentrated by adding 550µl of 70% ethanol, before column extraction. Phenolic phase locked under the gel of 2ml "PhaseLock" Heavy Gel tube, was discarded into special chloroform-containing waste. The aqueous/alcohol mixture was carefully transferred onto an RNeasy spin column and the tube was centrifuged and washed according to the manufacturer's instructions (details protocols provided in the RNeasy mini kit). An intermediate DNA digestion was carried out using a DNAase kit to reverse DNA (Qiagen, UK). The total RNA was eluted in 30µl of RNA-free water and stored at -80°C until further use.

2.2.2 RNA quantification and quality assessment

Total RNA concentration and quality were measured by absorbance of eluted RNA, using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, ThermoFisher Scientific, UK). Concentration and purity of RNA was established by measurement of absorbance at 260/280nm and 260/230nm. All RNA samples were

standardised to 100ng/µl, the RNA concentration required for reverse transcription as optimal in our laboratory.

2.3 Reverse transcription: complementary DNA synthesis

Synthesis of complementary DNA (cDNA) from RNA samples was performed using the Superscript® VILO™ synthesis kit (Invitrogen, Paisley, UK). The reaction was made to a final 1x VILO reaction mix with a total volume of 20µl, containing: primers, deoxynucleotide nucleoside triphosphates (dNTPs), $MgCl_2$ and ribonuclease inhibitors, 0.125x Superscript Enzyme Mix (reverse transcriptase enzyme) and 100ng RNA (Table 2-1). Samples were incubated at 25°C for 10 minutes (primer annealing phase), 42°C for 60 minutes (primer extension phase) and 85°C for 5 minutes (inactivation of reverse transcription phase) in a thermal cycler (MJ Research PTC 200 Thermal Cycler, BC-MJPC200, USA).

Before undertaking PCR analysis a cDNA standard curve was created to determine primer efficiency; standards were created using a 10-fold serial dilution from pooled concentrated RNAs. Controls included a human positive control (Applied Biosystems, UK) and 2 technical negative controls. A "NO RT" control, where the reverse transcriptase enzyme was omitted and replaced by 0.25µl of RNA free water, for identifying possible DNA contamination. A second "NEGATIVE" control, where the RNA was omitted and instead replaced by 1µl of RNA free water, allowing detection of contamination of reagents and/or primer dimer formation during PCR reactions. A "POSITIVE" control was also included, using a RNA sample that was known to contain cDNA encoding the gene of interest.

Reagent	Final concentration	Volume
5x VILO Reaction mix	1x	4µl
10x Superscript Enzyme Mix	0.125x	0.25µl
RNA (100ng/ml)	100ng	1µl
RNA free H_2O		14.75µl
Total Volume		20µl

Table 2-1 Protocol used for a single reaction for Reverse Transcription

2.4 Quantitative Real-Time (Taqman® PCR)

For this study the Taqman® Real-Time PCR technique was used. This method relies on the ability of *Taq* polymerase to cleave fluorescently labelled probes bound on a template cDNA sequence. In brief, Taqman® probes are oligonucleotides containing a fluorophore at one end and a quencher at the other end, the latter suppresses the fluorophore's fluorescence as long the quencher and fluorophore are in close proximity to each other (Figure 2-1-A). Following binding of the forward primer, elongation by Taq polymerase (polymerisation step, Figure 2-1-B) the polymerase reaches the binding site of the labelled probe, the 5'-3' exonuclease enzyme activity of Taq cleaves the probe from the DNA template Figure 2-1-C). Removing from the proximity of the quencher molecule resulting in fluorescent detection by the PCR machine (Figure 2-1-D). As each cycle results in release of fluorophore, the amount of fluorescence is proportional to the amount of cDNA in the initial reaction mix.

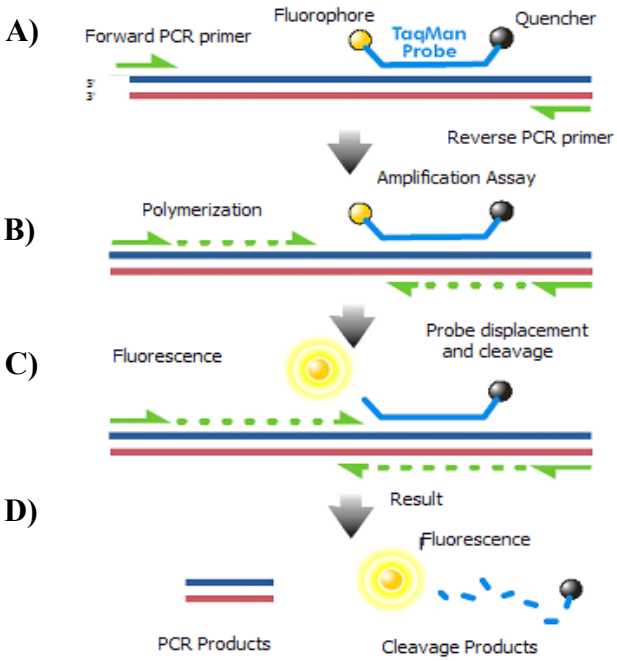

Figure 2-1 Taqman probe mechanism during quantitative PCR.
Single step described in Section 2.4. Image adapted from Agilent Technologies (2012).

2.4.1 Universal Probe Library (UPL™) and Taqman® Mastermix

The Universal Probe Library (UPL™, Roche, UK) contains 165 short hydrolysis probes labelled with FAM tag (6-carboxyfluorescein) on their 5'-end and with a dark quencher on their 3'-end. These probes consist of 8-9 nucleotides chosen because their sequences are present in the transcriptome of several species. The UPL™

probes have locked nucleic acids (LNAs), which have a protected ribose ring with a methylene bridge with the 2'-O atom connected to the 4'-C atom. LNAs are able to bind to their target sequences regardless of their short size, because they contain the common nucleobases (T, C, G, A, U and mC) that RNA and DNA contain.

In this study, primers were specifically designed by using the "Universal ProbeLibray Assay Design Center" (Table 2.2), and were purchased from Eurofins Genomics, Billingham, UK).

Gene name	Forward primer	Reverse primer	UPL probe number
Homo Sapiens tryptase alpha/beta (*TPSAB1*)	cctgcctcagagaccttcc	acctgcttcagaggaaatgg	20
Homo Sapiens chymase 1 (*CMA1*, set 1)	ttcacccgaatctcccatta	tcaggatccaggattaatttgc	81
Homo Sapiens chymase 1 (*CMA1*, set 2)	acggaactttgtgctgacg	ggctccaagggtgactgtta	4
Human Sapiens proteinase-activated receptor-2 (*PAR-2*)	gagccatgtctatgccctgt	cgatgcagctgttaagggtag	13
Homo Sapiens Histamine receptor H1 (*HR1*)	agaatcagacctgggtggaa	aatgagtctgaggctcccatag	68
Homo Sapiens Nuclear receptor subfamily 3, group C, member 1: glucocorticoid receptor (*GR* or *NR3C1*)	tccctggtcgaacagttttt	gctggatggaggagagctta	45
Human Sapiens Prostaglandin E receptor 2 (subtype EP2, *PTGER2*)	tggctatcatgaccatcacc	gtttcattcatatatgcaaaaatcgt	68
Homo Sapiens Oestrogen Receptor 1 (*ESR1* or *ERα)*	aaccagtgcaccattgataaaa	cctcttcggtcttttcgtatc	69
Human Sapiens Oestrogen Receptor 2 (*ESR2* or *ERβ*)	gctcctgtcccacgtcag	tgggcattcagcatctcc	62

Table 2-2 Table of oligonucleotide sequences and UPL probes used in RT-PCR analysis.

Reactions contained the qPCR Supermix with Premixed ROX kit (Invitrogen, UK). Taqman® mastermix (Table 2-3) and was plated in a 96-well MicroAmp Fast Optical reaction plate (Applied Biosystem, UK) with 14µl final volume in each well. Thereafter 1µl cDNA from the standard curve, controls and samples were added in duplicate to the individual wells. The qPCR reaction was carried out using Applied Biosystems 7900HT Fast real-time PCR system machine (Applied Biosystems, UK), with the following programme: 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute.

Reagent	Company	Final Concentration	Volume
Express Supermix (2x)	Invitrogen	1x	7.5µl
Forward Primer (20µM)	Eurofins	200nM	0.15µl
Reverse Primer (20µM)	Eurofins	200nM	0.15µl
Probe (10µM)	Roche	100nM	0.15µl
GAPDH or CYC (primers-probe mix, 20x)	ThermoFisher Scientific	1x	0.75µl
cDNA	---		1µl
RNA free H_2O	Qiagen		5.75µl for GOI 6.05µl for GAPDH or CYC
Total Volume			15µl

Table 2-3 Real-time PCR Taqman reaction mix.

2.4.2 qRT-PCR analysis

Amplification graphs were plotted using the detection of fluorescence and the cycle threshold (Ct) value, which was defined as the amplification cycle where the signal of detection was higher than the background noise levels. Notably Ct values are inversely proportional to the amount of targeted nucleic acid in the sample; a change of 1-fold in Ct values corresponds to a 2-fold change in the initial cDNA concentration and therefore gives indication of gene expression at mRNA level.

2.4.2.1 Primer amplification efficiency

In an ideal PCR amplification reaction duplication of the amount of RNA occurs every cycle, but contamination and a sub-optimal primer design can also interfere with the efficiency of the PCR reaction. Together with the R^2 value for the standard curve, efficiency (E) of all the primer sets was calculated by the formula $E = (10^{(-1/slope)}-1)$ x100 10-fold dilutions. E is expressed in percentage value, and in this study, ΔΔCt analysis was performed only for primer sets with an efficiency range of 95-105%. Otherwise, relative standard curve method of analysis was used.

2.4.2.2 Relative standard curve analysis

The relative standard curve (rSC, Figure 2-2) method determines the quantity of cDNA in each experimental sample using a standard curve equation and then comparing it to a calibrator sample. The standard curve is employed to deduce relative expression information for mRNA encoded by gene(s) of interest in samples. The values (x) of the standard curve are expressed a $\log_{10}$ of the mRNA concentration, then expressed to the power of 10 (10^x). The obtained values are normalized to the endogenous controls, such as GAPDH or CYC (ThermoFisher Scientific, UK). These absolute results are then converted into relative values expressed as ratio of sample x to the selected control sample.

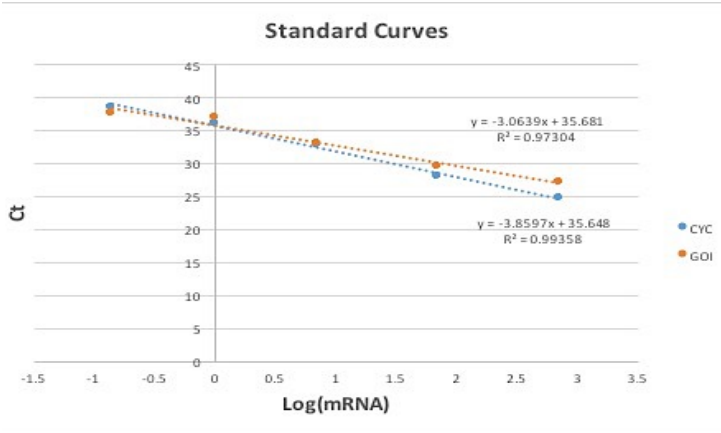

Figure 2-2 Example of standard curves and primer sets efficiency calculation.

The Ct value of each standard is graphed against the log10 of mRNA concentration both CYC (endogenous control) and gene of interest (GOI). The line formula is used for relative standard curve method analysis as explained in 2.4.2.2.

2.4.2.3 Statistical analysis

Statistical analysis was accomplished using GraphPad Prism 6.0 (GraphPad Software Inc., USA), using statistical tests as described in corresponding result chapters.

2.4.3 Separation of DNA in polyacrylamide gels

Electrophoresis with polyacrylamide gels is one of the most widely used tools in molecular biology. This technique is suitable for the separation of small pieces of DNA (from 1-1000 base pairs) because of the smaller pore size in a polyacrylamide matrix compared to those made of agarose. Double stranded real-time PCR products were run on Tri-borate-EDTA (TBE) gels (Novex pre-cast gel cassettes composition in Table 2-4, LifeTechnology, ThermoFisher, UK) in native (non-denaturing) conditions, using 1x TBE running buffer in a XCell SureLock™ Mini-Cell

(ThermoFisher Scientific, UK). DNA samples and ladder were prepared with Novex® Hi-Density TBE sample buffer (5x, Novex, Life UK) as described in Table 2-4.

Reagent	Volume
DNA sample	15 µl
Novex® Hi-Density TBE sample buffer (5x)	4 µl
Deionized water	1 µl
Total volume	20 µl
100bp DNA ladder (Invitrogen, Paisley, UK)	1µl
Novex® Hi-Density TBE sample buffer (5x)	2 µl
Deionized water	7 µl
Total volume	10 µl

Table 2-4 DNA samples and ladder preparation mix before electrophoresis.

Samples and a molecular size ladder (100bp DNA ladder, Invitrogen, Paisley, UK) were loaded onto the gel and the tank was filled with 200ml of 1x TBE buffer in the upper buffer chamber and 600ml in the lower buffer chamber. Gels were electrophoresed at 200V for 60 minutes with an expected current of between 10-18mA at the start and 4-6mA at the end of the run.

Once electrophoresis was complete, TBE gels were stained with 1x Sybr® Safe DNA gel stain for 30 minutes on a rotating platform (1:10000 dilution in 1x TBE running buffer; ThermoFisher Scientific, UK) before visualising the DNA band using a UV-transluminator (U:Genius, Syngene, UK).

2.5 Immunohistochemistry

Immunohistochemistry is a scientific method that visualises proteins in cells or tissue sections by employing an antibody that has specificity for specific antigenic epitopes within the target proteins.

2.5.1 Tissue fixation and processing

Due to the use of tissue from different species and diverse detection methods in this study, two main fixatives were used; human samples used for immunofluorescence staining where fixed in 4% neutral buffered formalin (Sigma Aldrich, UK) at 4°C for 16h and mouse samples used for toluidine blue staining (Section 5.4.5) were fixed in Carnoys' fixative (with 60% absolute alcohol – VWR Chemicals, UK, plus 30% chloroform and 10% acetic acid – Sigma Aldrich, UK) for 6h at room temperature (RT). After fixation, tissues were transferred into 70% ethanol for storage prior to paraffin embedding (performed by the SURF Histology facility in

the MRC Centre for Reproductive Health, the University of Edinburgh). 5µm sections were cut using a microtome (Leica Biosystems, RM2135, UK) and flattened in a 37°C water bath to allow the paraffin to stretch and remove folds (ThermoFisher Scientific, UK). The sections were then mounted on charged glass slides (Leica Biosystem Surgipath X-tra Adhesive, pink labelled for human tissues and white labelled for animal tissues, UK) and dried at 50°C overnight.

2.5.2 Dewaxing and tissue rehydration

To improve the antibody-antigen interaction within fixed tissue, sections were first deparaffinised (or dewaxed) and rehydrated. Dewaxing is accomplished by immersing the slides in xylene (VWR Prolabo, UK) for 10 minutes. Rehydration is characterized by immersing the slides in solutions of decreasing alcohol concentration (2 washes in 100% ethanol for 20 seconds each, then one wash each of 95% ethanol for 20 seconds, 70% ethanol for 20 seconds and tap water).

2.5.3 Antigen retrieval

Aldehyde-containing fixatives, such as 4% NBF, result in cross-linking of amino acids which may mask epitopes recognised by antigen formations. Therefore antigen retrieval was used to unmask the antigenic epitopes of interest (Figure 2-3).

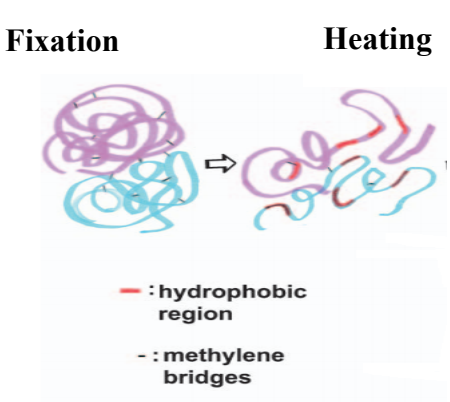

Figure 2-3 Schematic of heat-induced antigen retrieval.
After heating, methylene bridges produced by formalin fixation, are cleaved and the protein chains expose their hydrophobic sites. Image adapted from Emoto et al. (2005)

In this study, heat-induced epitope retrieval (HIER) that relies on both heat and high pressure was used. Briefly, after rehydration, the slides were placed in 1x pH 6 citrate retrieval buffer (1mM citric acid (mono hydrate)) in a decloaking chamber (BioCare Medical, Concord, USA). Maximum temperature reached during antigen retrieval step was 110°C.

2.5.4 Methanol-Peroxidase block

Slides were blocked with 3% hydrogen peroxide (H_2O_2; VWR Prolabo, UK) in methanol (VWR Prolabo, UK) on the rocking platform (at 3g/min, Heidolph Polymax 2040, Germany) for 30 minutes to prevent endogenous tissue peroxidases reacting with the chromogen substrate. Slides were then rinsed in tap water and washed twice with Tris Buffered Saline (TBS: 0.05M Tris-HCl, pH 7.4, 0.85% NaCl, Sigma Aldrich, UK) for 5 minutes.

2.5.5 Serum block

In order to prevent non-specific binding of the secondary antibody to the tissue sections, a blocking step with serum from the species that the secondary antibody was raised in is required. The serum reduces non-specific binding during the assay because it contains antibodies that bind to reactive sites on the tissue.

In this study, tissue sections were incubated with normal goat serum (NGS) buffer containing 20% NGS (Sigma Aldrich, UK) and 0.05% bovine serum albumin (BSA; Sigma-Aldrich, UK) in TBS for 30 minutes at room temperature.

2.5.6 Avidin-Biotin block

Chromogenic immunohistochemistry relies on the strong affinity between biotin and avidin molecules. To prevent avidin-peroxidase complexes reacting with endogenous biotin present in the tissue, instead of binding target protein-biotinylated secondary antibody complexes, an avidin-biotin block is required. Slides were blocked for 15 minutes with avidin, washed twice with TBS and incubated for 15 minutes with biotin (Avidin-Biotin Kit, Vector Laboratories, UK). Slides were again washed twice with TBS before adding the primary antibodies.

2.5.7 Primary antibody staining

Primary antibodies were diluted in NGS at optimized concentrations and left at 4°C for 16h in a humidity chamber. Appropriate controls were also included, such as negative control (primary antibody omission) and positive controls (tissues that strongly express the antigen of interest). The list of antibodies and dilutions used is shown in Table 2-5.

Primary Antibody	Species raised	Source	Clone	Concentration	Dilution	Secondary Antibody	Use
Tryptase	Rabbit	Abcam (ab134932)	EPR8476	3.428 mg/ml	1:500	1:500 - Goat anti-rabbit biotinylated (Vector, BA-1000)	DAB
Chymase	Mouse	AbSerotec (MCA1930T)	CC1	1.0 mg/ml	1:500	1:200 - Goat anti-mouse biotinylated (Vector, BA-9200)	DAB
Tryptase (1)	Rabbit	Abcam (ab134932)	EPR8476	3.428 mg/ml	1:300	1:200 - Goat anti-rabbit peroxidase (Abcam, ab7171)	IF
Tryptase (2)	Mouse	Dako (M7052)	AA1	1.0 mg/ml	1:3000	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF
Chymase	Mouse	AbSerotec (MCA1930T)	CC1	1.0 mg/ml	1:5000	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF
Human Oestrogen Receptor α (ERα)	Mouse	Vector (VP-614)	6F11	7.5 mg/ml	1:200	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF
Human Oestrogen Receptor β (ERβ)	Mouse	AbSerotec (MCA1974GA)	PPG5/10	1.0 mg/ml	1:200	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF
Human Progesterone Receptor (PR)	Mouse	Novacastra (NCL-PGR-312)	16	1.0-8.0 mg/ml	1:3000	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF
Human glucorticoid Receptor (GR)	Mouse	Novacastra (NCL-GCR)	4H2	25 µg/ml.	1:100	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF
Protease Activated Receptor 2 (PAR-2)	Mouse	Abcam (ab184673)	SAM11	1.0 mg/ml	1:500	1:200 - Goat anti-mouse peroxidase (Abcam, ab6823)	IF

Table 2-5 Primary antibodies, secondary antibodies and dilutions used in immunohistochemistry and immunofluorescence.

2.5.8 Secondary antibody incubation

Slides were washed for 5 minutes in 0.05% Tween in TBS (TBS-Tween, Tween: VWR Prolabo, UK) and then twice in TBS only before the secondary antibody blocking step. The secondary antibodies selected for immunofluorescence antigen detection were streptavidin-HRP for DAB immunohistochemistry or directly horseradish peroxidase conjugated (HRP) for immunofluorescence.

2.5.9 Antigen detection methods

2.5.9.1 DAB immunohistochemistry

Target antigen detection can be achieved by using 3,3'-diaminobenzidine (DAB), a chromogenic method. This method relies on the oxidation of DAB by antigen bound-antibody HRP complexes and generates a brown deposit at the site of antibody binding (Figure 2-4).

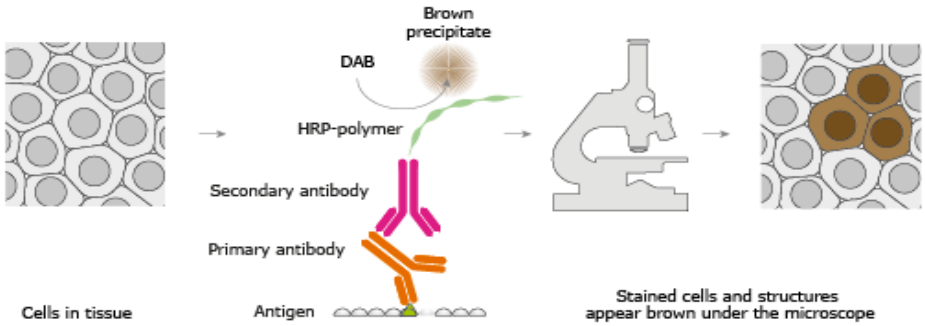

Figure 2-4 Schematic of DAB immunohistochemistry principle.
HRP-secondary antibody interacts with the DAB substrate and generates a brown precipitate in positive cells. Image adapted from the Human Protein Atlas (2016).

Streptavidin-HRP conjugated secondary antibodies (Vector Laboratories, UK) were diluted at 1:500 in TBS, and incubated on a section for 30 minutes. Slides were washed twice in TBS for 5 minutes and the DAB chromogenic substrate was applied to the tissue sections following the manufacturer's instructions. The chromogenic reaction was monitored microscopically and then stopped by immersing the slides in tap water. The tissues were counterstained for 2 minutes in Harris' haematoxylin (Leica Biosystems, UK), which turns the acidic substances, such as nucleic acids in the cell nuclei, blue. Slides were washed with tap water to remove any excess haematoxylin, and then immersed in 1% acid alcohol (CellPath, UK) for 20 seconds and in Scott's tap water (CellPath, UK) for 30 seconds. The tissue sections were dehydrated using increasing concentrations of ethanol (70%, 80%, 95% and absolute ethanol, 20 seconds each) before immersion in xylene (2x 5 minutes immersion steps; VWR Collection, UK). The slides were mounted using Pertex (Cell Path, UK) and a coverslip (VWR Collection, UK).

2.5.10 Immunofluorescence: tyramide signal amplification fluorescence system.

Immunofluorescence enables the detection of multiple target proteins in the same tissue section using a tyramide signal amplification (TSA™) system that allows the user to employ up to 1000-fold less concentrated primary antibodies as compared to chromogen immunohistochemistry.

The TSA reagent is a phenolic molecule that once activated by the horseradish peroxidase (HRP) (Figure 2-5-A) is transformed into a short-life compound (Figure 2-5-B). This is exceptionally reactive to free radical intermediates. These intermediate molecules can quickly react and bind to electron-rich-regions of proteins. HRP is conjugated to the secondary antibody, bound to the primary-target antigen complex (Figure 2-5-C).

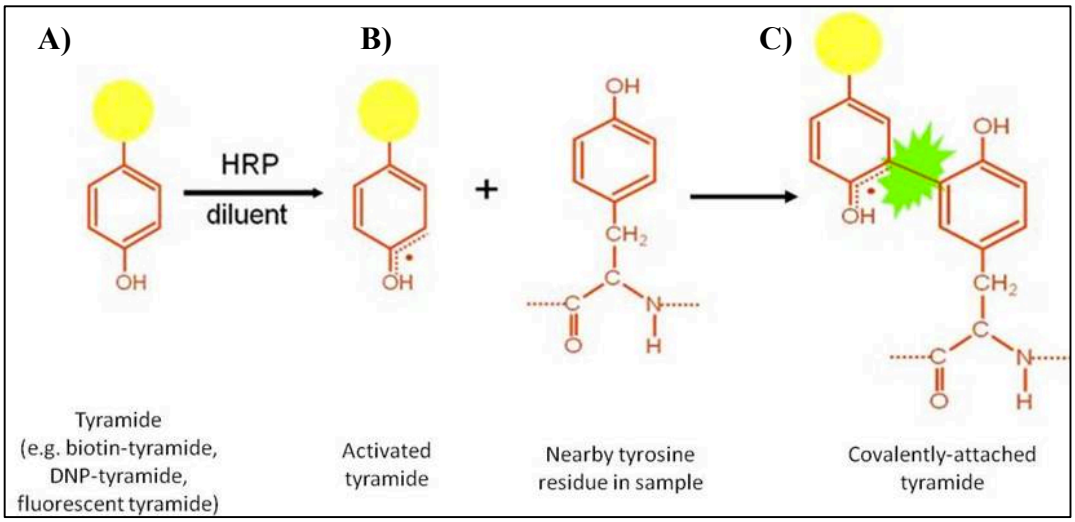

Figure 2-5 Tyramide signal amplification by covalent binding with electron-rich regions of protein of interest.
Chemical reaction explained in Section 2.5.10. Image taken from PerkinElmer (2015).

The covalent binding between tyramide and an antigen of interest releases fluorescence and it can be detected by using confocal microscopy. There are different fluorochromes with distinct wavelengths of excitation and emissions and minimal spectra overlap, that allows concurrent detection of different antigens. For this study fluorescein (green) 494nm/517nm (excitation/emission wavelengths) and cyanine 3 (red) 550nm/570nm were used.

Following the HRP-secondary antibody incubation, slides were washed in TBS-Tween and then TBS for 5 minutes each. The TSA system was used at 1:50 dilution following the manufacturer's instructions (TSA™, PerkinElmer, USA). TSA incubation was for 10 minutes in the dark, and was followed by two washes with TBS. Because TSA is light sensitive, the slides were then kept in the dark during all the following steps in the protocol to prevent photo-bleaching of the fluorochromes.

2.5.11 Double fluorescence

During the study, dual antigen detection was performed by immunofluorescence. After the first TSA fluorochrome incubation, usually fluorescein, tissue sections were incubated in TBS+3% H_2O_2 if the second primary antibody did not require any antigen retrieval, for example chymase antibody. Citrate antigen retrieval was performed for other second primary antibodies (PAR-2, ERα, ERβ, PR and GR) that required antigen retrieval.

Slides were washed twice in TBS and blocked with NGS for 30 minutes before incubating in the second primary antibody at 4°C for 16h. The subsequent steps in double IF protocol are as the aforementioned in Sections 2.5.8 - 2.5.10. The second TSA fluorochrome used was Cyanin 3.

2.5.12 Fluorescent nuclear counterstain

4',6'-diamidino-2-phenylindole (Dapi) was used as nuclear counterstaining on tissue sections. Dapi is a fluorescent dye that has high affinity for A-T-rich regions of DNA. Dapi is excited by UV light. Its excitation wavelength is 358nm; and it emits at 461nm in the blue/cyan spectrum.

Slides were incubated with Dapi (Sigma Aldrich, UK) at 1:500 dilution in 1xTBS for 10 minutes. Two 5 minute TBS washes were performed before mounting the slides with Permafluor™ (ThermoFisher Scientific, UK).

2.5.13 Imaging and analysis

Slides stained with DAB were scanned on an Axioscan Z1 (Zeiss, UK) for quantitative analysis with Zen Blue software (Zeiss, Germany).

Fluorescence slides (tissues and cells) were visualized on a Zeiss 710 confocal microscope (Zeiss, UK) and scanned for further full size image analysis, and both Zen Black and Zen Blue software were used for analysis.

2.6 Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) are quantitative immunoassays that detect target proteins in biological liquids/fluids. As with immunohistochemistry (Section 2.5) and Western blotting (Section 5.3.13), this method relies on the specific binding of antibodies to epitopes on target proteins. There are several different types of ELISA, but only two have been used in this study. These are: non-competitive two-site "sandwich" ELISA (Figure 2-6-A) and competitive ELISA (Figure 2-6-B).

2.6.1 Non-competitive two-site "sandwich" ELISA

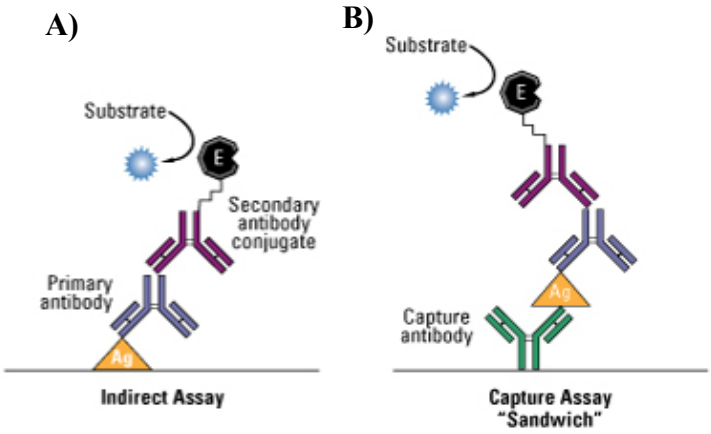

Figure 2-6 Illustration of Sandwich (A) and Competitive (B) ELISA methodologies. Image adapted from ThermoFisher (2011)

In the "sandwich" ELISA, the wells of a flat-bottomed 96-well microtiter plate are pre-coated with a capture antibody, which binds specifically to epitopes in the protein interest (Figure 2-6-A). Samples together with standard curve and blank are then added to the plate, and incubated for 1-2 hours. This incubation allows the first step of "sandwich" binding, between in the capture antibody and protein of interest present in the samples. A detection antibody is then added, which recognises a different epitope on the protein of interest. It forms the second part of "sandwich" with capture-protein complex. The detection antibody is conjugated with the HRP enzyme, or biotinylated, so that by the addition of streptavidin-HRP the amplification of a chromogenic change can take place. In these ELISAs the 3,3'-5,5'-tetramethylbenzidine (TMB) substrate is added to the wells and the chromogenic reaction starts by forming a blue product in the presence of the peroxidase enzymes.

The colour reaction is stopped by addition of an acid (usually, 2N sulphuric acid), it changes the TMB from blue into yellow. The optical density of each well is then measured using a microplate reader spectrophotometer set to the appropriate wavelength. The recorded signal is quantified using a standard curve. The amount of the protein of interest is proportional to the optical density of the TMB substrate.

2.6.1.1 Human tryptase and chymase "sandwich" ELISAs

In this study, the two-site "sandwich" ELISA was used for detection of human tryptase and chymase in peritoneal fluid (PF) from women with/without endometriosis. PF were stored at -80°C to preserve protein integrity and thawed immediately prior to the assay. Concentrations of tryptase and chymase were calculated using the pre-coated ELISA kits: SEB070Hu for tryptase (Cloud-Clone Corp., USA) and MBS2021042 for chymase (MyBioSource.com, USA), according to the manufacturers' instructions. Details of the protocol are reported in Section 4.3.9.

2.6.2 Competitive ELISA

Competitive ELISA relies on the principle that an unlabelled protein of interest present in the biological samples will compete for binding with enzyme-labelled antigen (HRP-conjugated) for a limited number of target antibody binding sites in the wells of the assay plate such that the HRP-conjugated antigen will bind to the target antibody only where the binding sites are not occupied by unlabelled protein of interest (Figure 2-6-B). As unbound reagents are removed by washing the amount of bound HRP in competitive ELISA is inversely proportional to the concentration of the levels of protein of interest in the sample. Two kits were used for investigating the levels of histamine in human peritoneal fluid: ENZ-KIT140 (Enzo Life Sciences, Switzerland) and CEA927Ge (Cloud-Clone Corp., USA). Details in Section 4.3.9.

2.6.3 ELISA data analysis

Data analysis was conducted by using GraphPad Prism 6.0 software to generate a four-parameter logistic (4-PL) curve fitting, the OD values of samples against the standards reads allowing calculation of the "unknown" value versus the "known" values of the standards. The data were statistically evaluated with GraphPad Prism 6.0 as explained in detailed in results Chapters 4 and 5.

2.7 Flow cytometry and fluorescence-activated cell sorting (FACS)

Flow cytometry is a technique that simultaneously evaluates multiple physical and chemical characteristics of single cells within a heterogeneous cell mixture, as they flow in a fluid stream through a beam of light.

This technology is based on three main systems: fluidics, optics and electronics. The first system carries particles to the laser beam for cellular interrogation, the second involves of a panel of lasers that illuminate the particles in the sample stream and of optical filters to direct the resulting light signals to the appropriate detectors. The third, system converts the detected light signals into electronic signals that can then be processed by a computer.

The main features measured include a particle's relative size, internal complexity, relative granularity and relative fluorescence intensity (Neuhoff, 1973). Cell size is determined by the forward scatter area (FSC-A), which is a parameter roughly proportional to the cell size. The internal complexity, such as nuclear structure and the presence of granules is discerned from the side scatter (SSC) (Figure 2-7).

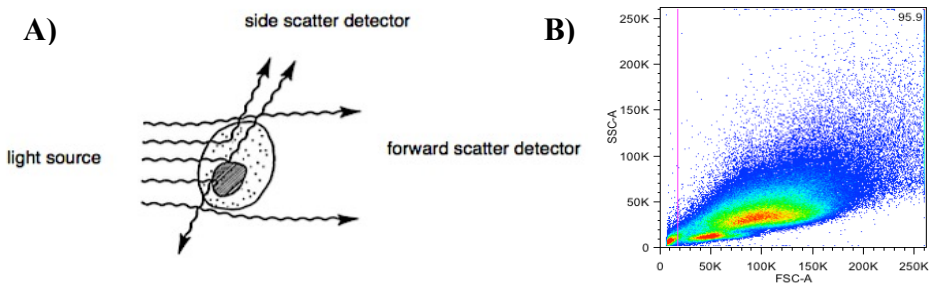

Figure 2-7 Light-scattering characteristics of cells going through the flow cytometer (A), FSC and SSC events collected by the detector and converted into a plot by the computer (B).
Figure 2-7-A adapted from BD Bioscience (2000).

Another important physical characteristic to consider is the pulse geometry, FSC-H (forward scatter height) versus FSC-A (Figure 2-8-C) but this can only be included in the analysis signals emitted by single cells. When cell clumps pass through the laser intercept (Figure 2-8-A), they take longer time compared to a single cell suspension (example 3µs for doublets vs 1µs single cell) and this affects the area of the signal as shown in Figure 2-8-B.

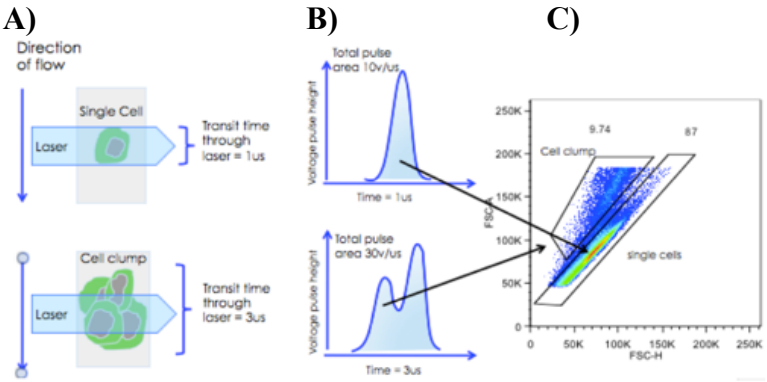

Figure 2-8 Differences in signal detection between single cells and cell aggregated going through a flow cytometer.
Image taken from Excyte Expert Cytometry (Excyte Expert Cytometry, 2012).

Relative fluorescence intensity can be used to describe cell populations by detection of different spectral emissions of cells going through the lasers following prior incubation with fluorescence-conjugated antibodies for the proteins of interests. In this study, flow cytometry and FACS were used to identify mast cells and other immune cell population isolated from human peritoneal fluids and mouse peritoneal lavages.

2.7.1 Sample compensation for multi-colour flow panel

Flow cytometry has continuously advanced over the last years. This has included an increasing number of colours that can be detected, up to 18 colours at the same time. This has allowed a more precise investigation of many cell types in samples with a mixed population and has also expanded the number parameters gathered simultaneously for each sample.

The most critical factor of a multi-coloured flow cytometry experiment is the spill over of emission spectra. This is often due to a large variability of intrinsic brightness among the fluorochromes frequently used, for example when spectra from one dye optically interfering with signals from another (Figure 2-9).

For examples, BV650 fluorochrome (detected in the blue laser) has an emission spectrum (650-775nm) that overlaps with APC (675-750nm, red laser).

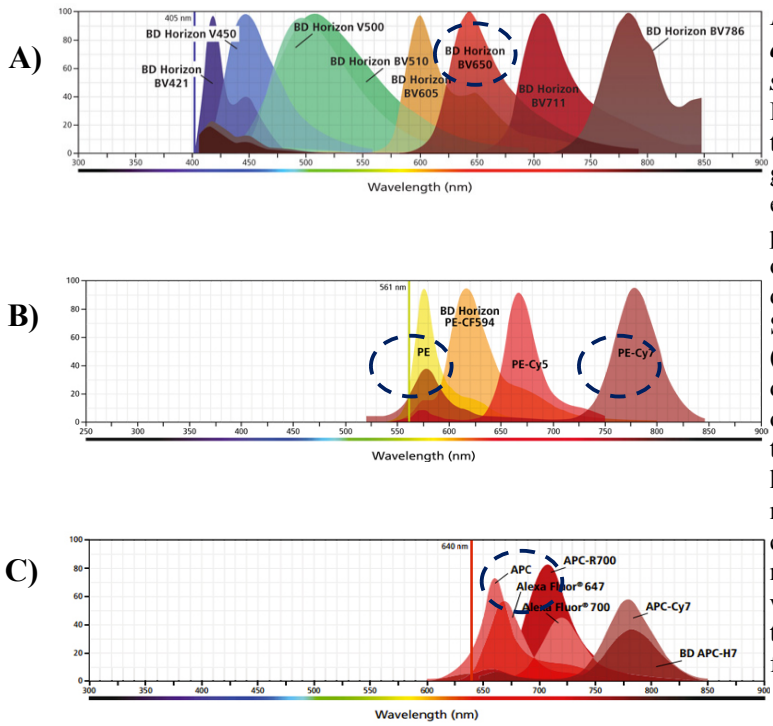

Figure 2-9 Fluorochromes excitation and emission spectra and possible spillovers. Knowledge of the spectra from the different lasers (A, blue; B, green/yellow; C, red laser) enables the operator to prevent possible overlaps between some of the antibodies included in the detection panel (Table 2-7). Some of the fluorochromes (BV650, PE, PE/CY7 and APC, dot circles) can influence each other's spectra and cause artefact the results. For example, BV650 has a spectrum range of 600-780 nm (excitation-emission) and it can interfere with PE (500-700 nm) due to their emission wavelengths that are very close to each other. Image adapted from BD Bioscience (2015).

Therefore, in a complex multi-parameter flow cytometry is crucial to perform both studies using unstained cells controls and compensation with beads. Using beads allows to the investigator save experimental cells, which are precious and are in limited number. Compensation beads are micro-particles, typically polystyrene, that are pre-coated with antibodies recognizing species-specific antibody light chains, IgG, κ light chain of mouse, rat and/or hamster (Figure 2-10).

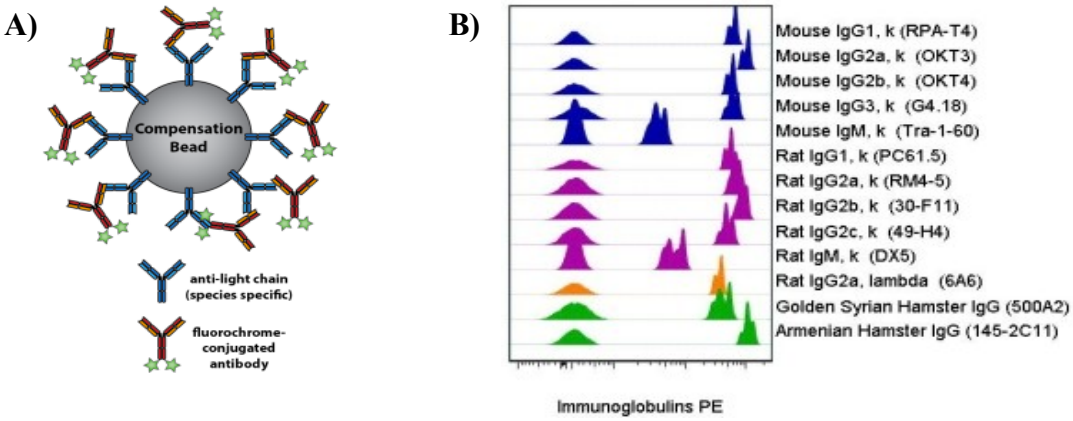

Figure 2-10 Compensation beads: their function and IgGs components used for flow cytometry. **A)** Compensation particle covered with anti-light chain antibody after binding with fluorochrome-conjugated detection antibody. Image adapted from UWCCC (2015). **B)** OneComp eBeads, light chain IgGs coated on the polystyrene micro-particles. Image adapted from Affymetrix (2016).

Beads are supplied with individual fluorochrome-conjugated antibodies for use as single-colour compensation controls. Each drop of beads contains two populations: a negative population that will not react with antibody and a positive population that will capture any mouse, rat or hamster antibody. When a fluorochrome-conjugated antibody is added to the beads, both positive and negative populations result.

The fluorescence minus one (FMO) control is another essential control that must be used to enable correct interpretation of flow cytometry data. It allows for detection and gating of cells in the context of a spread of data by a multiple fluorochromes. A FMO control consists in all the fluorochromes minus the one that is going to be measured. In this study, the human FMOs performed are shown in Table 2-6.

Fluorochrome / Antigen	PE	PE-CY7	BV650	APC
FcεRIα	-	c-kit	HLADR	CD163
c-kit	FcεRIα	-	HLADR	CD163
HLADR	FcεRIα	c-kit	-	CD163
CD163	FcεRIα	c-kit	HLADR	-

Table 2-6 FMOs used for flow cytometry analysis in the human study.
FcεRIα: high affinity IgE receptor I α, c-kit: stem cell factor receptor; HLADR: human leukocyte antigen – antigen D related; CD163: cluster of differentiation 16.

The FMOs ensured that any spread of the fluorochromes into the channel of interest was properly identified before running the cell samples, as illustrated in Figure 2-9.

2.7.2 Sample preparation

Following isolation of peritoneal fluid (PF) cells from human peritoneal fluids (Chapter 4, Section 4.3.3) and from mouse peritoneal lavages (Chapter 5, Section 5.4.6.1), samples stored -80°C were thawed at 37°C in a water bath, transferred into polystyrene flow cytometry tubes (BD Falcon, Oxford, UK) and washed with 3ml of filtered FACS buffer (phosphate-buffered saline without calcium and magnesium, PBS, ThermoFisher Scientific, Paisley, UK; 2% BSA, Sigma Aldrich, Dorset, UK). Samples were centrifuged for 5 minutes at 1000g at 4°C then cells were re-suspended in 1ml of FACS buffer and filtered to remove cell clumps, using filter polystyrene FACS tubes (BD Falcon, UK). Cell suspensions were counted and viability assessed using Countess II (as mentioned in Section 5.3.1).

2.7.3 Conjugated antibody staining

Samples were stained in 100µl of FACS buffer with the panel of anti-human antibodies, epitopes, concentrations and fluorochromes as illustrated in Table 2-7, anti-mouse antibodies are showed in Table 2-8. Single staining beads (eBioscience, Altrincham, UK) and FMOs were performed for compensation settings at the same time. Cell suspensions were kept in the dark, for 30 minutes (mouse cells) or for 1 hour

(human cells) on ice. Following incubation, samples were washed with 3 ml of FACS buffer and centrifuged for 5 minutes at 1000g at 4°C.

Primary Antibody	Species raised	Source	Clone	Fluorochrome	Cat. N°	Concentration	Dilution
CD45	Mouse	Invitrogen	HI30	PE-TexasRed	MHCD4517	69 µg/ml	1:100
CD3	Mouse	BioLegend	OKT3	BV711	317327	40-60 µg/ml	1:50
CD19	Mouse	BioLegend	HIB19	BV711	302245	40-50 µg/ml	1:50
CD56	Mouse	BioLegend	HCD56	BV711	318335	80-100 µg/ml	1:50
CD14	Mouse	BioLegend	M5E2	BV510	301841	150 µg/ml	1:50
CD11β	Mouse	BioLegend	ICRF44	BV605	301331	200 µg/ml	1:50
HLADR	Mouse	BioLegend	L243	BV650	307649	100 µg/ml	1:50
CD163	Mouse	BioLegend	GHI/61	APC	333610	80-100 µg/ml	1:50
c-kit	Mouse	BioLegend	AER-37 (CRA-1)	PE-Cy7	313211	200-400 µg/ml	1:50
FcεRIα	Mouse	BioLegend	104D2	PE	334609	25-100 µg/ml	1:50

Table 2-7 Anti-human conjugated antibodies used for flow cytometry and FACS studies.

Primary Antibody	Species raised	Source	Clone	Fluorochrome	Cat. N°	Concentration	Dilution
CD45	Rat	BD Horizon	30-F11	BV650	563410	0.2 mg/ml	1:200
CD3	Armenian Hamster	eBioscience	145-C11	PerCP-Cy5.5	45-0031-80	0.2 mg/ml	1:200
B220	Rat	BioLegend	RA3-6B2	FITC	103205	0.5 mg/ml	1:100
CD335	Rat	BioLegend	29A1.4	PE-Cy7	137617	0.2 mg/ml	1:200
CD11β	Rat	BioLegend	M1/70	APC-Cy7	101226	0.2 mg/ml	1:100
F4/80	Rat	eBioscience	BM8	PE-Cy5	15-4801-80	0.2 mg/ml	1:100
Ly6C/G (GR1)	Rat	BioLegend	RB6-8CS	PE-Dazzle	108451	0.2 mg/ml	1:100
c-kit	Rat	BD Biosciences	2B8	BV421	562609	0.2 mg/ml	1:200
FcεRIα	Armenian Hamster	BioLegend	Mar-1	PE	134308	0.2 mg/ml	1:200

Table 2-8 Anti-mouse conjugated antibodies used for flow cytometry studies.

2.7.4 Viability staining

To assess cell viability and to analyse live cells, the ability of cells to integrate the fluorescent DNA-binding probe Dapi was quantified. Dapi can be incorporated only if the cellular membrane has become porous, when the cells are non-viable. Dapi (D9542, Sigma Aldrich, UK) was added at 1:500 dilution to the cell suspension, 5 minutes before running the sample through the flow cytometer or sorter.

2.7.5 Equipment used for flow cytometry and FACS

Flow cytometry was achieved using a 6 laser BD Fortessa flow cytometer (BD, UK). FACS was performed by Centre for Inflammation Research Flow Cytometry facility team, using the BD Aria Fusion sorter (BD, UK).

2.7.6 Flow cytometry data analysis

Data gathered from flow cytometry and FACS were analysed using the software FlowJo 8.7 (Flowjo, LLC, USA). Statistical analysis was conducted using GrapPad Prism 6.0.

Chapter 3 Characterization of the phenotype of human uterine mast cells.

3.1 Introduction

Mast cells are derived from $CD34^+$/c-kit^+ pluripotent progenitors which reside in the bone marrow (Kirshenbaum et al., 1999). MC progenitors leave the bone marrow and enter the circulation, (Gurish and Austen, 2012), where they are recruited into peripheral tissues by chemokines secreted by stromal cells. Together with stem cell factor (SCF), a complex array of cytokines and a range of micro-environmental factors control the local development of progenitors into mature MCs (Valent et al., 1992). Expansion of tissue resident populations can also occur as MCs can be long-lived and may re-enter the cell cycle to undergo proliferation locally (Metcalfe et al., 1997). Mature mast cells are classified according to the proteases identified in granules; these are tryptase and chymase, which both are serine proteases. When secreted, these proteases contribute to host defence by promoting inflammation and tissue remodelling.

MCs are described as being MC_{TC}, which contain both tryptase and chymase and MC_T, which contain only tryptase (Collington et al., 2011, Wernersson and Pejler, 2014). MCs maturing in different tissue microenvironments vary widely in type and the amount of tryptase and chymase they contain (Caughey, 2007b). When MCs are activated they degranulate and the aforementioned proteases, together with other inflammatory meditators, stored in the cell's granules, are released into the extracellular environment by exocytosis (Tiwari et al., 2008, Lorentz et al., 2012). MC degranulation can occur in response to various stimuli in an IgE dependent or independent manner, as described by Galli et al (2011).

However, in addition to provoking the release of preformed granule components, MC activation also induces the *de novo* synthesis of many bioactive molecules, including leukotriens, prostaglandins and cytokines (Abraham and St. John, 2010).

The human endometrium undergoes cycles of cellular proliferation, differentiation, and secretory activity during each menstrual cycle (Johannisson et al.,

1987). In the absence of embryo implantation, the upper functional layer of the endometrium breaks down and is shed at menstruation (Jabbour et al., 2006). This monthly tissue remodelling process is regulated by changes in cyclical ovarian steroid hormones (Salamonsen and Lathbury, 2000, Kelly et al., 2001b, Maybin and Critchley, 2015). Endometrial tissue contains stromal, epithelial, and endothelial cells, as well as a diverse population of immune cells, such as T cells, neutrophils, macrophages and mast cells (MCs) (Evans and Salamonsen, 2012a, Thiruchelvam et al., 2013). Inflammatory cells represent a significant percentage of the total cell population within the endometrial stroma and their numbers fluctuate representing 10-15% of cells in the proliferative phase, 20-25% of cells in the late secretory phase, and up to 40% of cells immediately before menstruation (Kamat and Isaacson, 1987, Salamonsen and Woolley, 1999). Leukocyte recruitment (uterine natural killer cells (uNKs) and macrophages) is most striking during the final stages of the menstrual cycle, with numbers rising rapidly during the late secretory phase to support the maturation of the tissue during decidualization. Notably, the majority of neutrophils influx immediately prior to menses (Salamonsen and Lathbury, 2000, Evans and Salamonsen, 2012a).

There is evidence that mast cells are part of the inflammatory cell population in the uterine environment. Uterine MCs are predominantly present in the basal layer and myometrium, they are reported not to change in number across the menstrual cycle and they are considered to be tissue resident cells (Mori et al., 1997b). Few studies have reported that uterine MCs fit the common phenotypic classification: they may be both MC_T and MC_{TC}, with the tryptase only phenotype reported to be predominant in the functional layer (Jeziorska et al., 1995). MCs are usually considered as being in a quiescent state and only reported to be highly activated, in an IgE-independent manner, during the mid secretory phase (oedematous state) and immediately before and during menstruation (Jeziorska et al., 1995, Sivridis et al., 2001). MCs with low granule content, suggesting prior activation, have been observed within the endometrium at the premenstrual stages (Sivridis et al., 2001). MCs have been implicated in the active biochemical process of extracellular matrix (ECM) degradation during menstruation and implantation because they are able to release precursors of metalloproteinases (MMP-2, MMP-9) and precursors of collagenase (Lees et al., 1994).

Tryptase, a mast cell specific protease, is able to cleave substrates including its receptor PAR-2 (protease-activated receptor 2), which is expressed on several cell types including immune cells, fibroblasts and endothelial cells (Fiorucci and Ascoli, 2004, Ossovskaya and Bunnett, 2004). There is evidence of PAR-2 expression in the human endometrium throughout all phases of the menstrual cycle and after the establishment of pregnancy (Hirota et al., 2005). It has been reported that PAR-2 activation leads to *in vitro* proliferation of endometrial stromal cells and interleukin-8 release from both cultured endometrial stromal and epithelial cells (Hirota et al., 2005). These findings suggest that MCs are involved in both endometrial tissue remodelling and leukocyte recruitment in the final stages of the menstrual cycle.

Moreover, other studies have shown that MCs are critical during blastocyst implantation due to their ability to secrete histamine: a mediator that alters vascular permeability and also induces stromal decidualization via histamine receptor 1 (HR1) (Liu et al., 2004, Szewczyk et al., 2006). Blockage of MC degranulation or ablation of mast cells from the maternal interface resulted in impaired implantation and delayed development of the implants, altering trophoblast invasion in rodent models (Salamonsen et al., 1996, Woidacki et al., 2013b). Furthermore, the release of histamine and serotonin has been linked to uterine contractility, linked to the location of MCs within smooth muscle fibres in the myometrium (Rudolph et al., 1993, Bytautiene et al., 2004, Bytautiene et al., 2008). Smooth muscle cells are a source of stem cell factor (SCF), essential for MC progenitor maturation and MC survival (Nilsson et al., 1994b, Metcalfe et al., 1997).

Female sex hormones, such as oestradiol and progesterone, have long been suspected to have an effect on MC behaviour, due to the fact that many MC-related pathophysiological conditions have a higher prevalence in females rather than males (Narita et al., 2006). Studies in non-reproductive tissue systems and using the HMC-1 cell line (human mast cell line, (Butterfield et al., 1988)) reported that MCs express the oestrogen receptor α isoform (ERα) and the progesterone receptor (Nicovani and Rudolph, 2002, Zaitsu et al., 2007, Jensen et al., 2010). Alongside mRNA expression, authors showed that MCs can be rapidly stimulated to degranulate by oestradiol via ERα (Zaitsu et al., 2007).

3.1.1 Summary

Mast cells have been documented to be part of the leukocyte population in the human uterus, in both the endometrial and myometrial compartments. Only Jeziorska et al (1995) have investigated the tryptase/chymase phenotype of uterine MCs across the different phases of the menstrual cycle. In their studies they used single colour staining for endometrial proteases by staining serial sections.

Another important aspect to take into consideration is that MC development is specifically influenced by the microenvironment into which the cell progenitors migrate. The human endometrium is a specialised tissue environment, not comparable with others, which may have a unique impact on MC maturation and behaviour.

With these observations in mind, this study was designed to elucidate the phenotype of mast cells in the unique microenvironment of the human uterus by investigating the expression of serine proteases and sex hormone receptors throughout the menstrual cycle.

3.2 Aims of the Chapter

1. To investigate the spatial and temporal localization of uterine mast cells.
2. To investigate the phenotype of the uterine mast cell subtypes and their environmental specific expression of steroid hormone receptors.

3.3 Material and methods

3.3.1 Human tissue resources

Uterine samples were obtained from women undergoing total abdominal hysterectomy for non-malignant gynaecological conditions. Endometrium from the functional layer was collected by using a suction "pipelle" endometrial sampling device, and "full thickness" samples were collected after the hysterectomy was performed. The "full thickness" uterine sample includes the endometrium from the endometrial-myometrial junction to the uterine lumen (Figure 3-1).

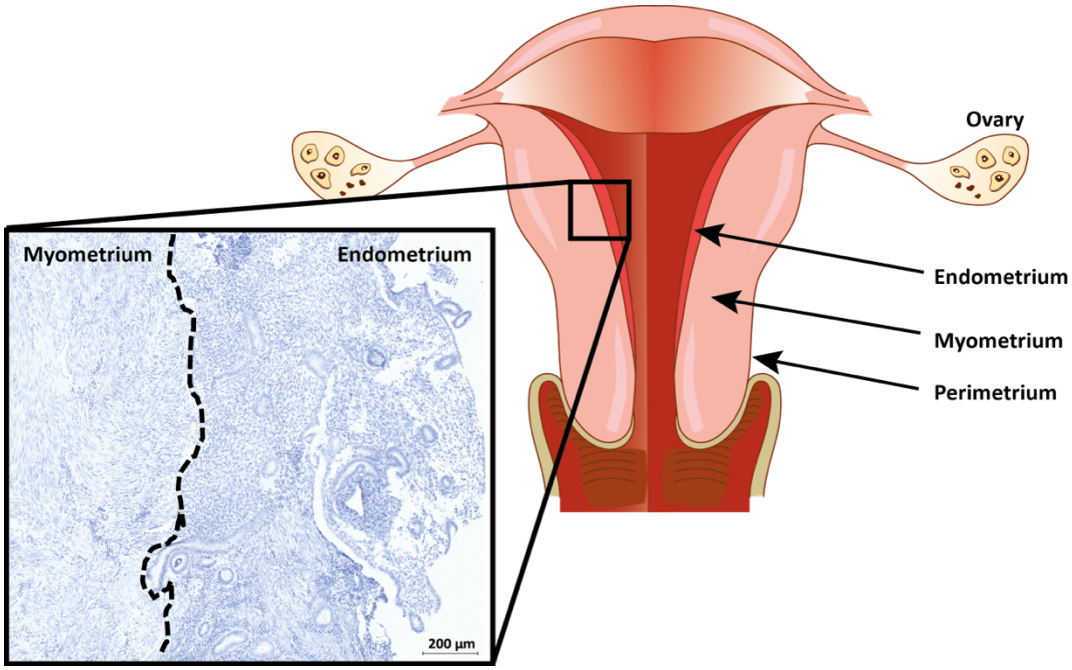

Figure 3-1 Endometrial-myometrial junction in uterine full-thickness biopsies.
The insert represents a haematolyxylin staining of a representative sample of full thickness biopsy, with the myometrial layer (left), endometrial layer facing the uterine cavity lumen. Image adapted from the original made by Mr Grant, graphic designer, MRC Centre for Reproductive Health, University of Edinburgh.

Patients were aged between 25-50 years (average of 39.8 years), had regular menstrual cycles and had not taken any exogenous hormones in the three months prior to surgery. Stage of the menstrual cycle was confirmed by histo-pathology and oestradiol and progesterone blood levels at the time of biopsy. Critical inclusion criteria were the absence of pelvic pain, such as dysmenorrhea, absence of fibroids or presence of small fibroids only (<3 cm). Decidual samples were collected during selective termination of pregnancy between 9-12 weeks of gestations. Local ethics committee approval was granted and written patient consent was obtained prior to

tissue collection by dedicated research nurses (Ethical approval was held by Professor H.O.D. Critchley: 10/S1402/59 and 16/ES/0007).

Experimental design and sample resources are illustrated in Table 3-1

Unique No	Serum E_2 (pg/ml)	Serum P_4 (ng/ml)	H&E Stage	Age	Average Menstrual cycle length	Surgical procedure	UF	Polyps	HMB	Endo	Pain	Use
7108	805	2.89	P	42	25	TAH	✗	✗	✓	✗	✗	IHC
5433	103.57	2.47	P	39	29	TAH	✗	✗	✓	✗	✓	IF
7815	3.3	<1	P	42	n.a.	Endometrial sampling	✗	✓	✗	✗	✗	RNA
5323	107	0.94	P	45	30	TAH	✗	✗	✓	✗	✗	IHC IF
5491	185	9.42	P	40	22	TAH	✗	✗	✗	✗	✗	RNA
7680	215	<3	P	36	28	TAH	✗	✗	✓	✗	✗	IF
7151	245	2.7	P	36	n.a.	Insert LNG-IUS	n.a.	✗	✗	✗	✗	RNA
7541	295	4.02	P	33	31.5	LapSter	✗	✗	✗	✗	✗	RNA
5342	318	2.6	P	45	21	VagHyst	✗	✗	✓	✗	✗	IHC IF
5144	357	1.8	P	41	30	LapSterRev	n.a.	✗	✗	✗	✗	RNA
5021	428	6.4	P	42	30	LapSter	n.a.	✗	✗	✗	✗	RNA
7728	444	<3	P	42	30	Hysteroscopy	✗	✗	✗	✗	✗	RNA
5612	472	2.33	P	37	28	LapSter	n.a.	✗	✗	✗	✗	RNA
5312	565	5.49	P	42	21	TAH	✗	✗	✓	✗	✗	IHC IF
5007	570	3.7	P	44	27	TAH	✗	✗	✓	✗	✗	IHC IF
7854	571	10.2	P	47	21	TAH	✗	✗	✓	✗	✗	IF
7903	628	3.2	P	40	23.5	TAH	✗	✗	✓	✗	✗	IF
7283	679.57	4.46	P	40	28	LapSter	n.a.	✗	✗	✗	✗	RNA
7115	921	7.89	P	44	28	TAH	✗	✗	✓	✗	✗	IF
5201	943	3.24	P	39	26.5	LapSter	n.a.	✗	✗	✗	✗	RNA
5616	1178	2.2	P	44	26.5	LapSter	✗	✗	✗	✗	✗	RNA
5074	988	34.1	ES	35	28	LapSter	✗	✗	✗	✗	✗	RNA
5158	604	42.5	ES	39	26	LapSter	✗	✗	✗	✗	✗	RNA
5037	592	46.1	ES	32	30.5	LapSter	✗	✗	✗	✗	✗	RNA
5067	514	22.8	ES	44	28	Polypectomy	✗	✓	✗	✗	✗	RNA
7294	504.29	72	ES	40	28	LAVH	✗	✗	✓	✗	✗	IHC
5541	244	51.75	ES	38	28	TAH	✗	✗	✓	✗	✗	IHC
5014	407	44.4	ES	36	28	LapSter	✗	✗	✗	✗	✗	RNA
7210	289.28	89.93	ES	33	28	VagHyst &PFR	✗	✗	✗	✗	✗	IHC IF
7882	338	28.5	ES	43	28	VagHyst &PFR	✗	✗	✗	✗	✗	IHC IF
5300	398	63	ES	35	30	Labial Cyst	✗	✗	✗	✗	✗	RNA
7248	549.91	88	ES to MS	32	28	TAH	✗	✗	✗	✗	✗	IHC IF
5211	502	43.2	ES to MS	43	24.5	STAH	✗	✗	✓	✗	✗	IF
3155	351	23.1	ES to MS	32	28	TAH	✗	✗	✓	✗	✓	IF
6412	460	32.5	MS	39	30	Ovarian Cystectomy	✗	✗	✗	✗	✗	RNA
5624	457	25.83	MS	30	28	Polypectomy	✗	✓	✗	✗	✗	RNA
7384	331	83.5	MS	42	24.5	TAH	✗	✗	✓	✗	✗	IHC IF
5690	323	38.6	MS	39	27.5	TAH	✓	✗	✗	✗	✗	RNA
7730	312	43.2	MS	39	29	Polypectomy	✗	✗	✗	✗	✗	RNA

7105	305	91.05	Mid Secretory	41	28	TAH	✗	✗	✓	✗	✓	IHC
7941	271	33.4	MS	46	28	TAH	✓	✗	✗	✗	✗	RNA
6359	203.3	16.63	MS	42	28	LapSter	n.a.	✗	✗	✗	✗	RNA
5607	38.2	7.09	MS to LS	38	25	TAH	✗	✗	✓	✗	✗	IF
5743	132	6.4	LS	47	28	TAHBSO	✓	✗	✓	✗	✗	IF
5135	602	14.7	LS	44	28	TAHBSO	✓	✗	✓	✗	✗	IF
7817	8.4	2.3	M	42	29.5	Endometrial sampling	✓	✗	✗	✗	✗	RNA
5316	371	7.54	M	40	28	LAVH	✗	✗	✗	✗	✗	RNA
7229	175.87	2.55	M	40	28	TAH	✗	✗	✓	✗	✗	IF
7909	242	<3	M	35	24.5	STAH	✗	✗	✓	✗	✗	IF
5609	196	8.21	M	38	30	Ovarian Cystectomy	✗	✗	✗	✗	✗	RNA

Table 3-1 Sample information and experimental application of tissue resources used in Chapter 3. Tissues were collected under Ethical approval 10/S1402/59 and 16/ES/0007. UF: uterine fibroids, HMB: heavy menstrual bleeding, Endo: endometriosis, n.a.: not applicable, TAH: total abdominal hysterectomy, STAH: subtotal abdominal hysterectomy, VagHyst: vaginal hysterectomy, PFR: pelvic floor repair, LAVH: laparoscopically assisted vaginal hysterectomy, LNG-IUS: levonorgestrel-releasing intrauterine system, LapSterRev: laparoscopic sterilization reversal. IHC: immunohistochemistry, IF: double immunofluorescence.

3.3.2 Tissue processing

Tissue samples were either placed into 4% neutral buffered formalin (NBF) for future tissue processing and paraffin embedding (Section 2.5.1) or into RNAlater for subsequent RNA extraction (Section 2.2).

3.3.3 RNA extraction from endometrial biopsies

Total RNA was extracted from homogenized endometrial tissue biopsies (20mg/sample) using TriReagent (Sigma Aldrich, UK) and the RNA fraction separated from tissue proteins using chloroform (Sigma Aldrich, UK). The RNA solution was transferred into an RNeasy spin column and manufacturer's instructions were followed (Qiagen RNA extraction kit, UK). DNA was digested using a DNase kit (Qiagen, UK) and total RNA was eluted in 30µl of RNA-free water. Samples were stored at -80°C for a minimum of 16h before measuring RNA concentration and quality with the Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, UK). RNA concentration was standardised to 100ng/µl in RNAse-free water prior to cDNA preparation.

3.3.4 Preparation of cDNA

Reverse transcription of RNA samples was accomplished by using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen, UK) in an adaptation of the manufacturer's protocol. In brief, each reaction included a final concentration of 1x VILO mastermix, 0.125x SuperScript enzyme and 100ng RNA in a total volume of 20µl. Sample were run at 25°C for 10 minutes, 42°C for 60 minutes and at 85°C for 5 minutes.

3.3.5 Quantitative Real Time PCR (Taqman® method)

Quantitative PCR was used for this study; principles of the method are explained in Section 2.4. Each reaction mix was prepared using qPCR Supermix with premixed ROX kit (Invitrogen, UK). The qPCR amplification reaction was carried out using the Applied Biosystems® 7900HT Fast Real-time PCR system using the reaction mix illustrated in Section 2.4.1. Primer sets were designed using the Roche Universal Probe Library (UPL, sequences are described in Table 3-2). Primers were purchased from Eurofins (Eurofins Genomics, UK) and the probes from the Roche UPL Human set (Roche Applied Science, UK). Samples were assayed in duplicate and run at 95° for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for a minute.

Gene name	Forward primer	Reverse primer	UPL probe number
Homo Sapiens tryptase alpha/beta (*TPSAB1*)	cctgcctcagagaccttcc	acctgcttcagaggaaatgg	20
Homo Sapiens chymase 1 (*CMA1*)	ttcacccgaatctcccatta	tcaggatccaggattaatttgc	81
Human Sapiens proteinase-activated receptor-2 (*PAR-2*)	gagccatgtctatgccctgt	cgatgcagctgttaagggtag	13
Homo Sapiens Histamine receptor H1, (*HR1*)	agaatcagacctgggtggaa	aatgagtctgaggctcccatag	68

Table 3-2 Table of oligonucleotide sequences used in RT-PCR analysis.

3.3.5.1 Relative mRNA expression and statistical analysis

PCR analysis was conducted using the standard curve method (Section 2.4.2.2) and compared to tonsil control mRNA (ASD-0088, Applied StemCell, Menlo Park, USA), a positive control for mRNA expression of mast cells mediators.

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, USA). Data are presented as median. One-way ANOVA was used, and Kruskal-Wallis was performed as a secondary test with Dunn's multiple comparisons test. Criterion for significance was $p<0.05$.

3.3.6 Immunohistochemistry

Immunohistochemistry was carried out on "full thickness" (uterine lumen to endometrial-myometrial junction) human uterine sections to establish the presence and localization of mast cells in the different tissue layers constituting the uterus: myometrium, basal endometrium and functional endometrium. Uterine biopsies were fixed in 4% NBF, embedded in paraffin wax and cut in 5µm sections. After dewaxing and tissue rehydration, sections were either exposed to antigen retrieval treatment using 1mM Citrate buffer (pH 6) when staining for tryptase, or were directly blocked in methanol peroxide for 30 minutes at RT when staining for chymase. All slides were then blocked in normal goat serum (NGS, 20% serum, 0.05% bovine serum albumin BSA in TBS, Sigma Aldrich, UK) for 30 minutes at RT, then an avidin-biotin block applied (Vector Laboratories, UK). Sections were incubated overnight at 4°C with the primary antibody (tryptase and chymase at the concentration of 1:500, details in Table 3-3). After primary antibody incubation, slides were incubated with secondary antibodies at 1:200 dilution (Table 3-3) for 1h at RT. Streptavidin-HRP was added before performing antigen detection with the 3,3'-diaminobenzidine substrate (DAB, Vector laboratories, UK). Slides were counterstained with haematoxylin and mounted with pertex (CellPath, UK) Slides were then scanned using the Axioscan Z1 (Zeiss, Germany).

Primary Antibody	Species raised	Source	Clone	Dilution	Secondary Antibody
Tryptase	Rabbit	Abcam (ab134932)	EPR8476	1:300	1:500 - Goat anti-rabbit biotinylated (ab7055)
Chymase	Mouse	AbSerotec (MCA1930T)	CC1	1:500	1:500 - Goat anti-mouse biotinylated (ab6822)

Table 3-3 Primary antibodies used for DAB immunohistochemistry.

3.3.7 Immunofluorescence

Detection of colocalised target antigens was accomplished by using the tyramide signal amplification system (TSA™, details in Section 2.5.10).

3.3.7.1 Image analysis and mast cell quantification using Fiji software.

DAB single stained images were acquired on the Axioscan Z1 and converted to Tiff files before using Fiji image analysis software (Schneider et al., 2012). The region of interest (ROI) was manually drawn (illustrated in Figure 3-2-A), to distinguish endometrial and myometrial layers. The two different areas were established by examining nuclei shape and differences in cellular density between the endometrial and myometrial compartments. Due to sample variability, select fields of view were not chosen, instead the whole area of endometrial and myometrial tissue was taken into account. The total ROI's area (was then cut from the original image and measured in pixels (Figure 3-2-B,C), converted into μm^2 based on the characteristic of

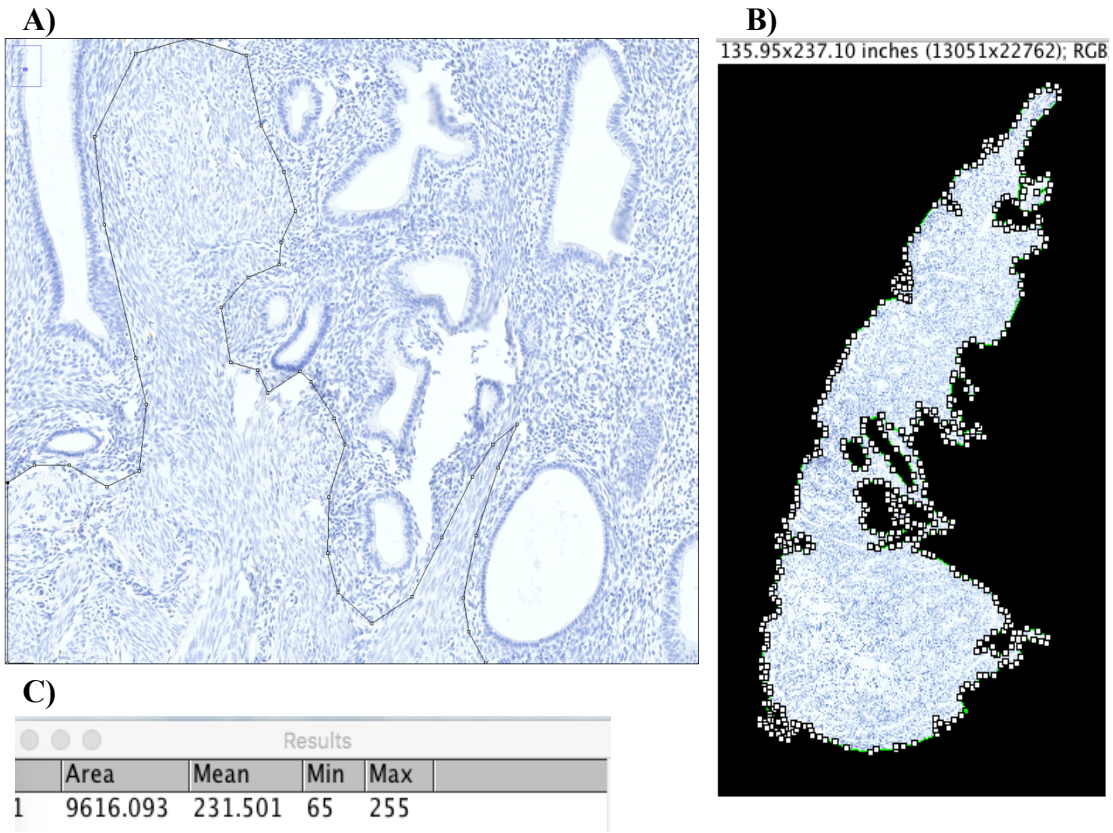

Figure 3-2 Region of interest establishment by manual selection. **A)** Example of manual selection of the endometrial compartment from the myometrium, based on nuclei density, in a uterine full thickness image. **B)** Separated endometrial after ROI selection, **C)** Table with pixel measurement of selected ROI area.

the optical objective and camera installed on the slide scanner (information was available in the czi file, and accessed using Zen Blue software). 1µm corresponded to 45.45 pixels.

DAB positive cells (brown stained) were quantified using the "cell counter plug-in", and manually selected throughout the ROI, as in the example shown in Figure 3-3-A. Figure 3-3-B illustrates the Cell Counter plug-in window, where positive cells were counted. Values were expressed as the total number of positive cells (tryptase or chymase single staining) in the ROI.

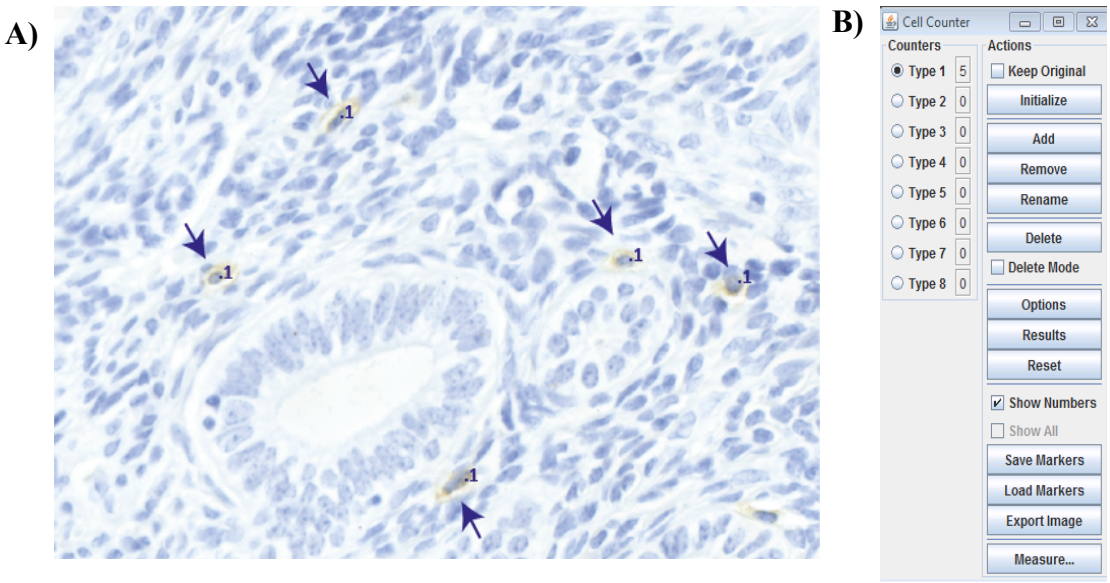

Figure 3-3 Mast cell quantification with Cell counter Fiji Plug-in.
A) High magnification of region of interest (ROI) with counted cells, **B)** Cell counter window with accounted positive cells. Blue arrows indicate DAB positive mast cells.

As a confirmation of quantification using DAB staining, images using double immunfluorescence images of representative samples were also quantified. A set area was drawn in myometrium and endometrium for each sample, fluorescent channels were colour deconvoluted for single staining quantification with Fiji software (Figure 3-4).

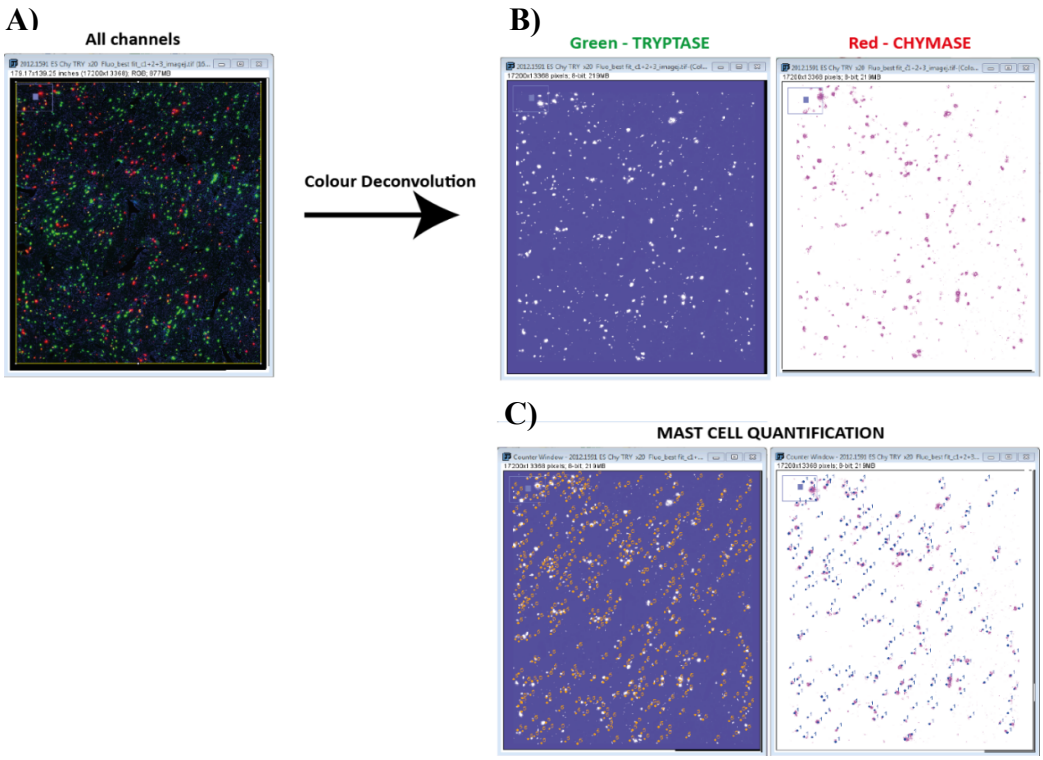

Figure 3-4 Immunofluorescence quantification after colour decovolution with Fiji software. **A)** Double immunofluorescent region of interest (ROI). **B)** Colour deconvoluted image, into green channel and red channel. **C)** Quantification of staining both in the green (tryptase) and in the red channel (chymase), using Fiji Cell Counter Plug-in.

3.4 Results

3.4.1 Concentration of mRNAs of mast cell specific proteases and receptors in the human endometrium.

Gene expression analysis was carried out on RNA extracted from tissue homogenates of human endometrium and decidua 9-11 week gestation to identify dynamic changes in the expression of genes encoding mast cell specific serine proteases, histamine receptor 1 (HR1) and protease-activated receptor 2 (PAR-2) (Figure 3-5).

Concentrations of messenger RNA encoded by *TPSAB1* (gene for tryptase α and β isoforms) remained unchanged throughout the different phases of the menstrual cycle; *TPSAB1* mRNA was significantly downregulated in first trimester decidua when compared to expression levels at the menstrual stage. Similarly, *CMA1* (chymase) mRNA concentrations did not change during the menstrual cycle, while lower levels were detected in first trimester decidua samples however this was not significant.

The mast cell mediators: histamine, tryptase and chymase may act through different receptors. In the current study the pattern of expression of HR1 and PAR-2 were selected for further investigation. *HR1* mRNA concentrations did not show any alteration during the phases of the menstrual cycle, whilst *HR1* was significantly downregulated in the decidua samples compared to menstrual phase samples. Interestingly, *PAR-2* mRNA concentrations were apparently higher during the early and mid-secretory phases when compared to proliferative and menstrual phase of the cycler and early pregnancy decidua, although they did not reach statistical significance.

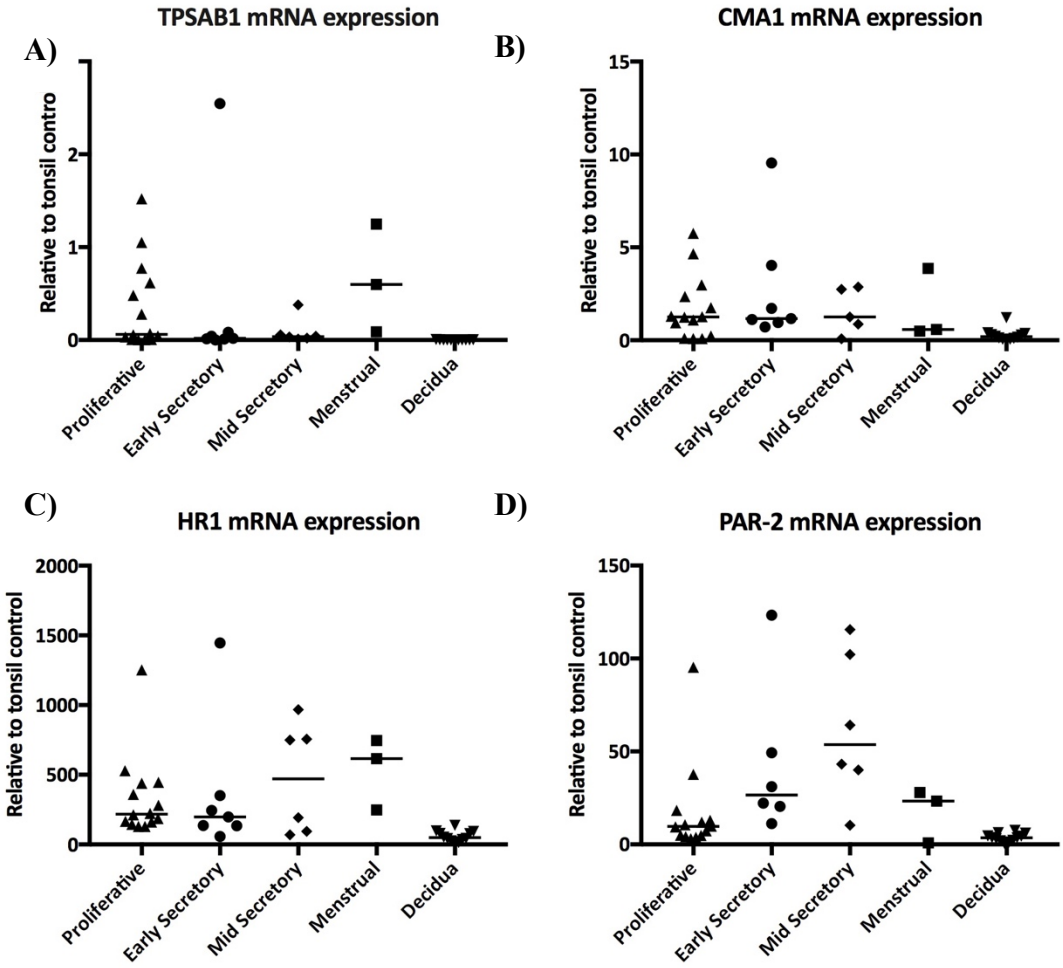

Figure 3-5 Expression of mRNAs encoding mast cell specific proteases, histamine receptor 1 and protease-activated receptor 2 in human endometrial and decidual biopsies.
A) *TPSAB1* mRNA expression was not significantly changed across the menstrual cycle. Interestingly mRNA levels were apparently lower during mid secretory phase and significantly decreased in decidua samples when compared to menstrual phase tissue. **B)** *CMA1* mRNA expression was not significantly changed across the menstrual cycle. mRNA levels during early secretory phase showed a trend of increase when compared to proliferative phase levels. **C)** *HR1* receptor mRNA expression remain unchanged in endometrial biopsies and it was significantly downregulated in decidua samples. **D)** *PAR-2* receptor mRNA expression was increased during early and secretory phases. Single dots represent different samples, and data are expressed as median. Statistical test used was One-way ANOVA, Kruskal-Wallis post-test, *$p<0.05$. Proliferative phase n=14, Early Secretory phase n=7, Mid Secretory phase n=6, Menstrual phase n=3 and Decidua n=12.

Expression of PAR-2 was also confirmed at the protein level (Figure 3-6). Immunofluorescence showed that PAR-2 was expressed in the cytoplasm of both endometrial stromal and epithelial cells. More intense immuno-staining was detected in glandular epithelial cells during early and mid-secretory phases, which is at the time when it is reported that epithelial cell proliferation is occurring.

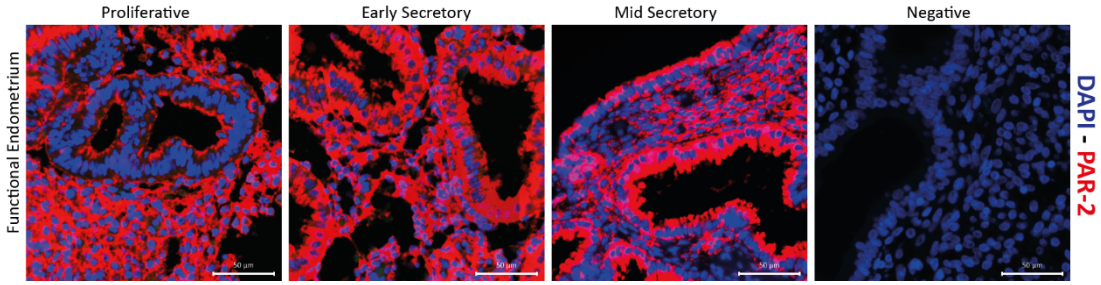

Figure 3-6 PAR-2 protein immunolocalization in endometrial functional layer during the proliferative, early and mid secretory, and menstrual cycle stages.
PAR-2 protein is present in the human endometrium; being located both in the epithelial and stromal cell compartments. Epithelial expression is apparently higher during both early and mid-secretory stages when compared to proliferative phase tissue. This finding confirms the significant PAR-2 mRNA upregulation during secretory phases, see Figure 3-5. Proliferative n=3, Early Secretory n=2, and Mid Secretory n=3. Negative control: omission of primary antibody.

3.4.2 Localization of mast cells in "full thickness" sections of human uterus.

Staining for individual proteases was performed using DAB detection on serial sections and cells were quantified following the methods as outlined by Jeziorska et al (1995). Results were generated using 10 "full thickness" uterine samples (endometrial-myometrial junction to uterine lumen): 5 proliferative phase and 5 secretory phase (representative sample staining showed in Figure 3-7). Counting of DAB positive cells showed inconsistent results regarding numbers of MCs of each phenotype during the proliferative phase. Quantification revealed that 2/5 proliferative endometrial and 3/5

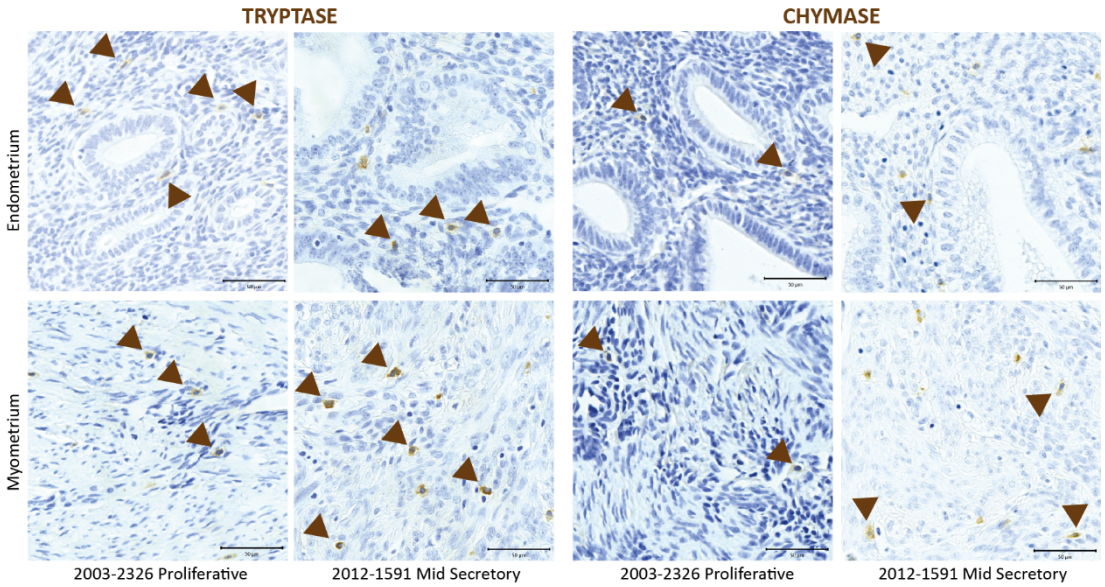

Figure 3-7 Single DAB staining for tryptase and chymase on representative samples of human uterus from across the menstrual cycle.
Brown triangles indicate DAB positive cells.

proliferative myometrial samples had a higher percentage of chymase positive cells compared to tryptase positive cells (see Table 3-4, Table 3-5).

Endometrium		MC-Tryptase positive	MC-Chymase positive	Ratio Try:Chy	Percentage Tryptase positive	Percentage Chymase positive
Sample code	**Histological stage**					
2001-1145	**Proliferative**	0	4	0:4	0%	100%
2003-1213	**Proliferative**	33	66	33:66	33%	67%
2003-1814	**Proliferative**	7	2	7:2	78%	22%
2003-2326	***Proliferative***	*42*	*4*	*42:4*	*95.5%*	*4.5%*
2008-2333	**Proliferative**	5	1	5:1	83%	17%
2007-1323	**Mid Secretory**	23	15	23:15	60.5%	39.5%
2007-1321	**Early Secretory**	1	1	1:1	50%	50%
2012-1591	***Early Secretory***	*141*	*188*	*141:188*	*43%*	*57%*
2005-1949	**Early Secretory**	7	2	7:2	78%	22%
2005-1076	**Mid Secretory**	142	40	142:40	78%	22%

Table 3-4 Quantification of tryptase and chymase single DAB staining in endometrial serial sections. Rows shaded in grey identify samples with abundant MCs used for further study.

Myometrium		MC-Tryptase positive	MC-Chymase positive	Ratio Try:Chy	Percentage Tryptase positive	Percentage Chymase positive
Sample code	**Histological stage**					
2001-1145	**Proliferative**	12	53	12:53	18%	82%
2003-1213	**Proliferative**	713	1010	713:1010	41%	59%
2003-1814	**Proliferative**	14	17	14:17	45%	55%
2003-2326	***Proliferative***	*119*	*67*	*119:67*	*64%*	*36%*
2008-2333	**Proliferative**	340	73	340:73	82%	18%
2007-1323	**Mid Secretory**	235	133	235:133	64%	36%
2007-1321	**Early Secretory**	166	84	166:84	66%	34%
2012-1591	***Early Secretory***	*463*	*225*	*463:225*	*67%*	*33%*
2005-1949	**Early Secretory**	233	65	233:65	78%	22%
2005-1076	**Mid Secretory**	856	723	856:723	54%	46%

Table 3-5 Quantification of tryptase and chymase single DAB staining in myometrial serial sections. Rows shaded in grey identify samples with abundant MCs used for further study.

To further quantify protease expression by immunohistochemistry, representative samples (grey rows in Table 3-4, Table 3-5) were used for dual immunofluorescence staining for tryptase and chymase. Data showed that uterine MCs were more likely to express tryptase in their granules (over 55% of the total number of mast cells, Table 3-6) than chymase. This finding confirms that both MC_T and MC_{TC} phenotype are present in the human uterus. Comparing results with those in tables 3-5, 3-6 it is also apparent that double immunofluorescent staining using the tyramide system is a more reliable method than analysing single DAB stains on serial sections when quantifying MCs in uterine tissue.

Samples		MC-Tryptase positive	MC-Chymase positive	Ratio Try/Chy	Percentage Tryptase positive	Percentage Chymase positive
Sample code Histological stage	**Tissue**					
2003-2326	Endometrium	59	48	59:48	55%	45%
Proliferative	Myometrium	166	78	166:78	68%	32%
2012-1591	Endometrium	213	68	213:68	76%	26%
Mid Secretory	Myometrium	384	213	384:213	64%	36%

Table 3-6 Quantification of double staining for mast cell tryptase and chymase

3.4.3 Immuno-profiling of uterine mast cell subtypes based on the expression of proteases: tryptase and chymase

The location and phenotype of uterine MCs in functional and basal endometrium, and in myometrium, were extensively investigated using double immunofluorescence staining for tryptase and chymase on "full thickness" biopsies obtained from normally cycling women (Figure 3-8).

MCs were rare in the stroma of the functional endometrium, independent of the phase of the cycle. In the basal layer, MCs were mainly detected at the myometrial-endometrial junction. Basal endometrial MCs were seen in the stromal compartment close to epithelial cells and endothelial cells. In some areas of the basal endometrium mast cells were observed in clusters. In the myometrium, mast cells were located in, and between, muscle bundles and closed associated with blood vessels.

The activation/degranulation state of uterine MCs appeared to be cycle stage specific. For example, during the proliferative phase, immunostaining of MCs showed a round phenotype with proteases with the cytoplasm suggesting they were not activated hence no extracellular release of tryptase or chymase was detected (Figure 3-8-C). Evidence of MC activation within the human uterus was detected during the early secretory phase, a stage known to be associated with stromal oedema. This observation suggests that MCs undergo activation/degranulation at specific phases of the menstrual cycle and that they are intimately involved with cellular changes in the stromal compartment.

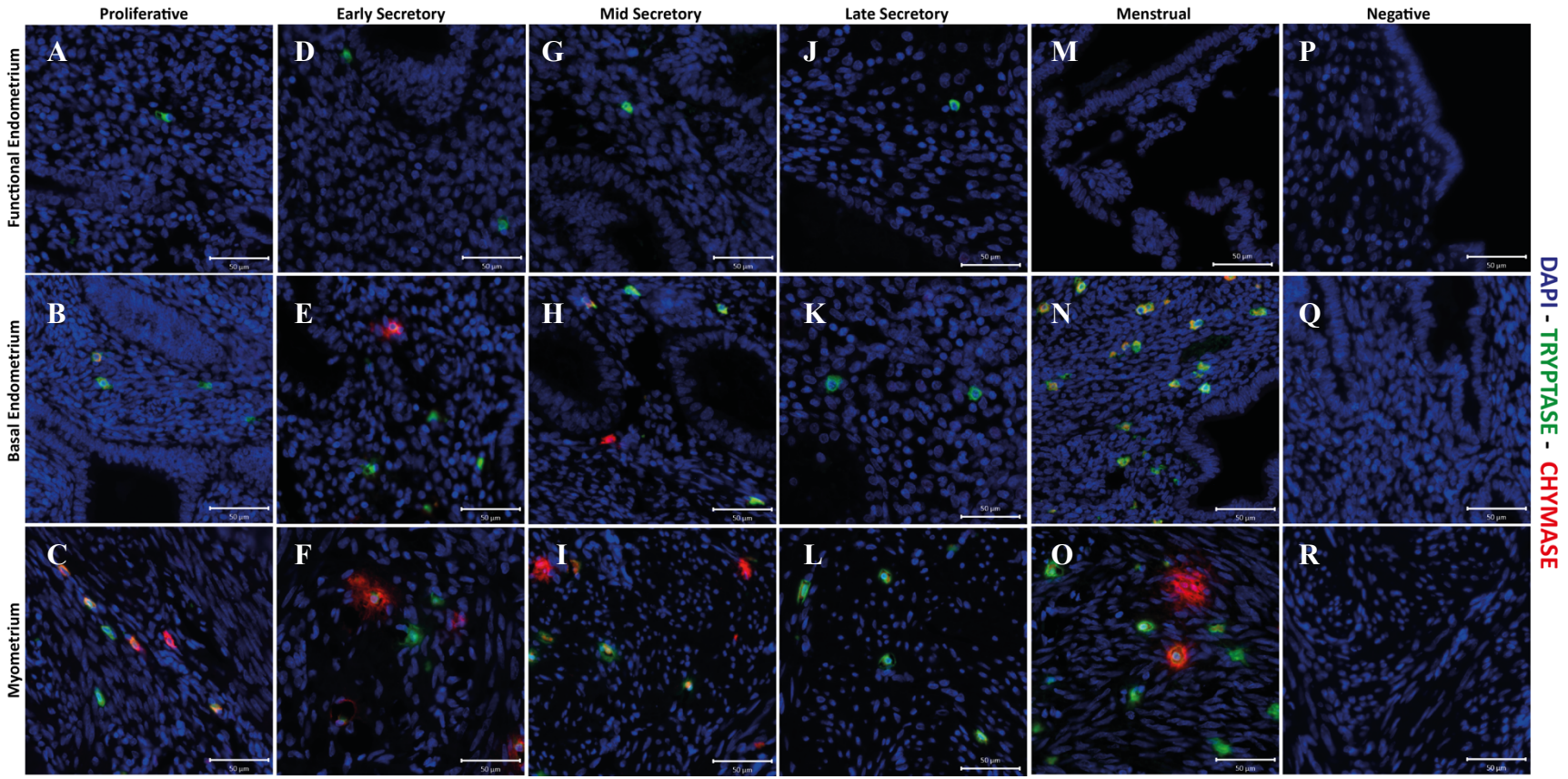

Figure 3-8 Identification of mast cell subtypes in full thickness uterine biopsies across the menstrual cycle by tryptase and chymase immunolocalization.
A-B-C) Myometrium, basal and functional endometrium during proliferative phase (P); **D-E-F)** Early secretory phase (ES); **G-H-I)** Mid secretory phase (MS); **J-K-L)** Late Secretory phase (LS); **M-N-O)** Menstrual phase (M); **P-Q-R)** Negative control (omission of primary antibody). Double immunofluorescence has revealed the presence of three uterine mast cell subtypes, single tryptase, single chymase and double tryptase-chymase positive cells. (P n=4, ES n=4, MS n=2, LS n=3, M n=2).

3.4.3.1 Proliferative phase

Uterine MC phenotype and activation profiles were further analysed in each phase of the menstrual cycle. Interestingly, the uterine MC phenotype differed between the three compartments of the "full-thickness" uterine tissue sections as revealed using confocal imaging (Figure 3-9). In the myometrial layer, MCs expressed tryptase and chymase with different intensities and multiple cell phenotypes were identified: MC_T with high tryptase and no chymase; MC_{TC} with high tryptase and low chymase, defined by intensity of the fluorescent signal; or MC_{TC} with low tryptase and high chymase (Figure 3-9).

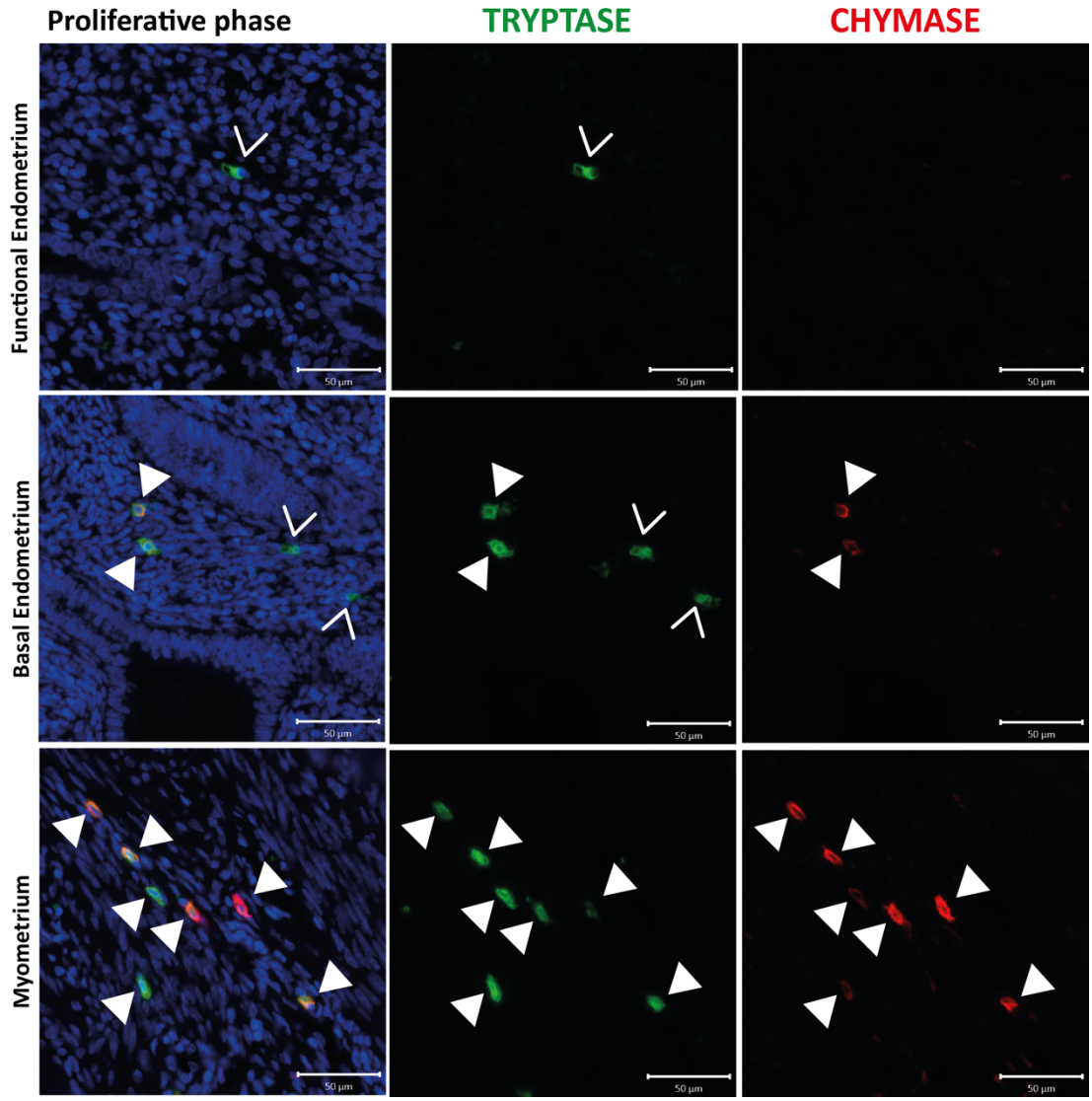

Figure 3-9 Mast cell subtypes and activation state in the proliferative phase endometrium.
Single fluorescence channels show the different mast cell subtypes in a uterine "full thickness" section during proliferative phase of the menstrual cycle. The myometrial compartment shows three different mast cell subtypes: tryptase single positive, chymase single positive and tryptase and chymase double positive. Basal endometrial mast cells are tryptase single positive and double positive, instead functional endometrium MCs are fewer in number and show a tryptase only phenotype. The MC activation profile during proliferative phase looks quiescent; proteases are retained in the cytoplasm. (n=4) (White triangles: MC_{TC} cells; white Vs: MC_T cells; white arrows: MC_C cells).

In contrast, basal endometrial MCs expressed tryptase only or high tryptase/low chymase in their granules. Endometrial MCs in the functional layer were only detected by immunostaining for tryptase, revealing the MC_T phenotype (white Vs in Figure 3-9).

3.4.3.2 Early secretory phase

The MC activation profile changed during the early secretory phase (Figure 3-10). MCs appeared activated in both basal endometrium and myometrial layers. In

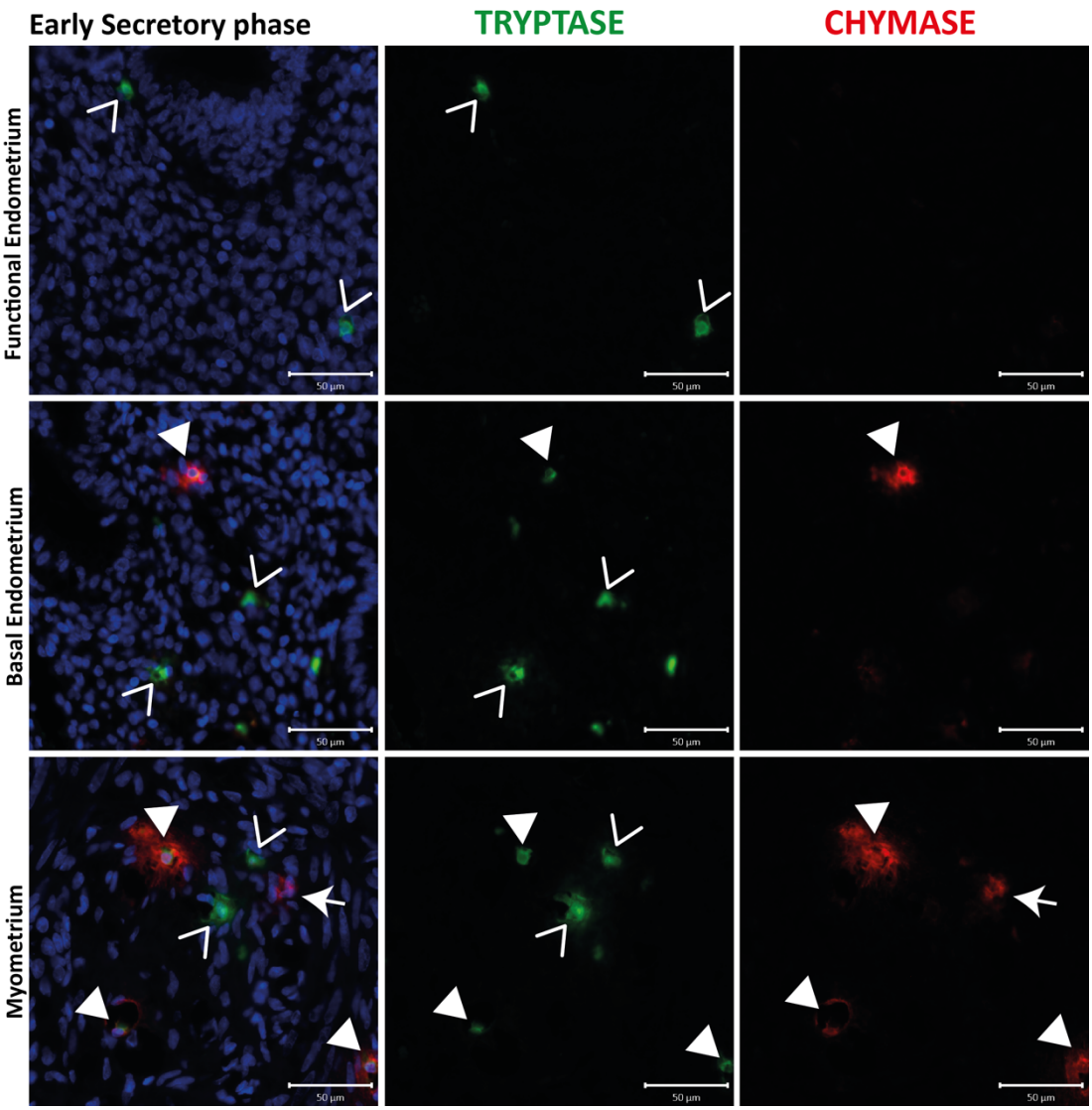

Figure 3-10 Mast cell subtypes and activation state during the early secretory phase.
Single fluorescence channels show the different mast cell subtypes in a uterine full thickness section during the early secretory phase. Myometrial compartment shows three different mast cell subtypes: tryptase single positive, chymase single positive and tryptase and chymase double positive. Basal endometrial mast cells are tryptase single positive and double positive, instead functional endometrium MCs are fewer in number and show a chymase negative phenotype. MCs during the early secretory phase appeared to be activated, releasing both proteases from the cytoplasm. (n=4) (White triangles: MC_{TC} cells; white Vs: MC_T cells; white arrows: MC_C cells).

the myometrium cells appeared to have released both tryptase and chymase, whereas in the basal endometrium chymase alone was released. Additionally, the uterine MC phenotype was either MC_T or low tryptase/high chymase MC_{TC}.

3.4.3.3 Mid secretory phase

During the mid-secretory phase, MCs appeared to degranulate in the myometrial compartment, with both tryptase and chymase released. In contrast, MCs in the endometrium were in a resting state with proteases retained in their cytoplasm.

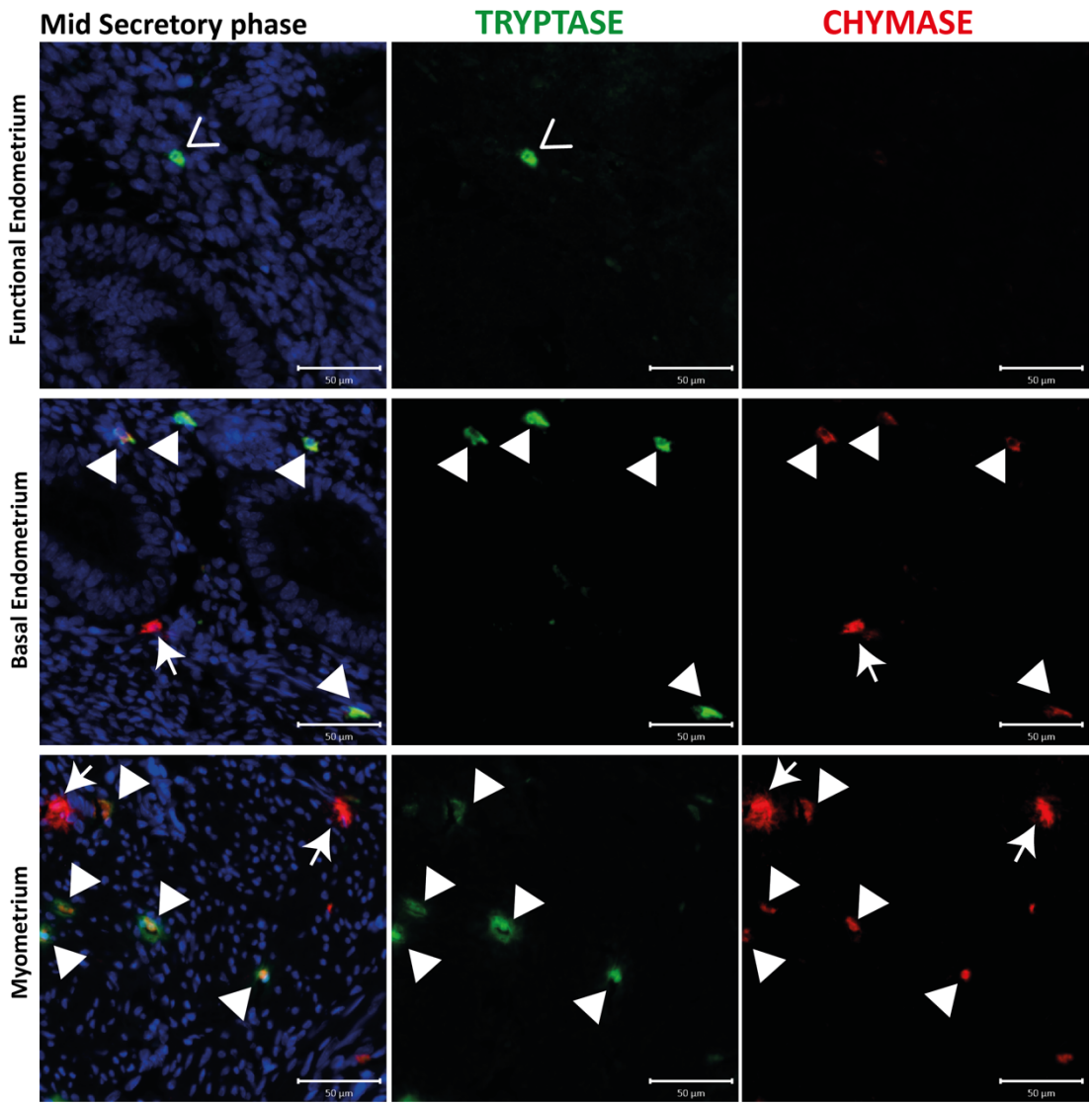

Figure 3-11 Mast cell subtypes and activation state during the mid secretory phase.
Myometrial compartment shows three different mast cell subtypes: tryptase single positive, chymase single positive and tryptase and chymase double positive. Basal endometrial mast cells are tryptase single positive and double positive, instead functional endometrium MCs are fewer in number and show a chymase negative phenotype. MCs during the early secretory appeared to be activated in the myometrium and basal endometrium, releasing both proteases from the cytoplasm. (n=3) (White triangles: MC_{TC} cells; white Vs: MC_T cells; white arrows: MC_C cells).

Regarding the phenotypic profile, a different subtype of MCs was also detected, together with the common $MC_{TC,}$ in basal endometrium and myometrium: MC_C only (white arrows in Figure 3-11) were present. In accordance with other findings (Mori et al., 1997b), MCs found in the stroma of the functional endometrium, showed MC_T phenotype (white triangle).

3.4.3.4 Late secretory

During the late secretory phase (Figure 3-12), activated MCs were detected only in the myometrium, with extra-cellular tryptase located outside the cytoplasmic

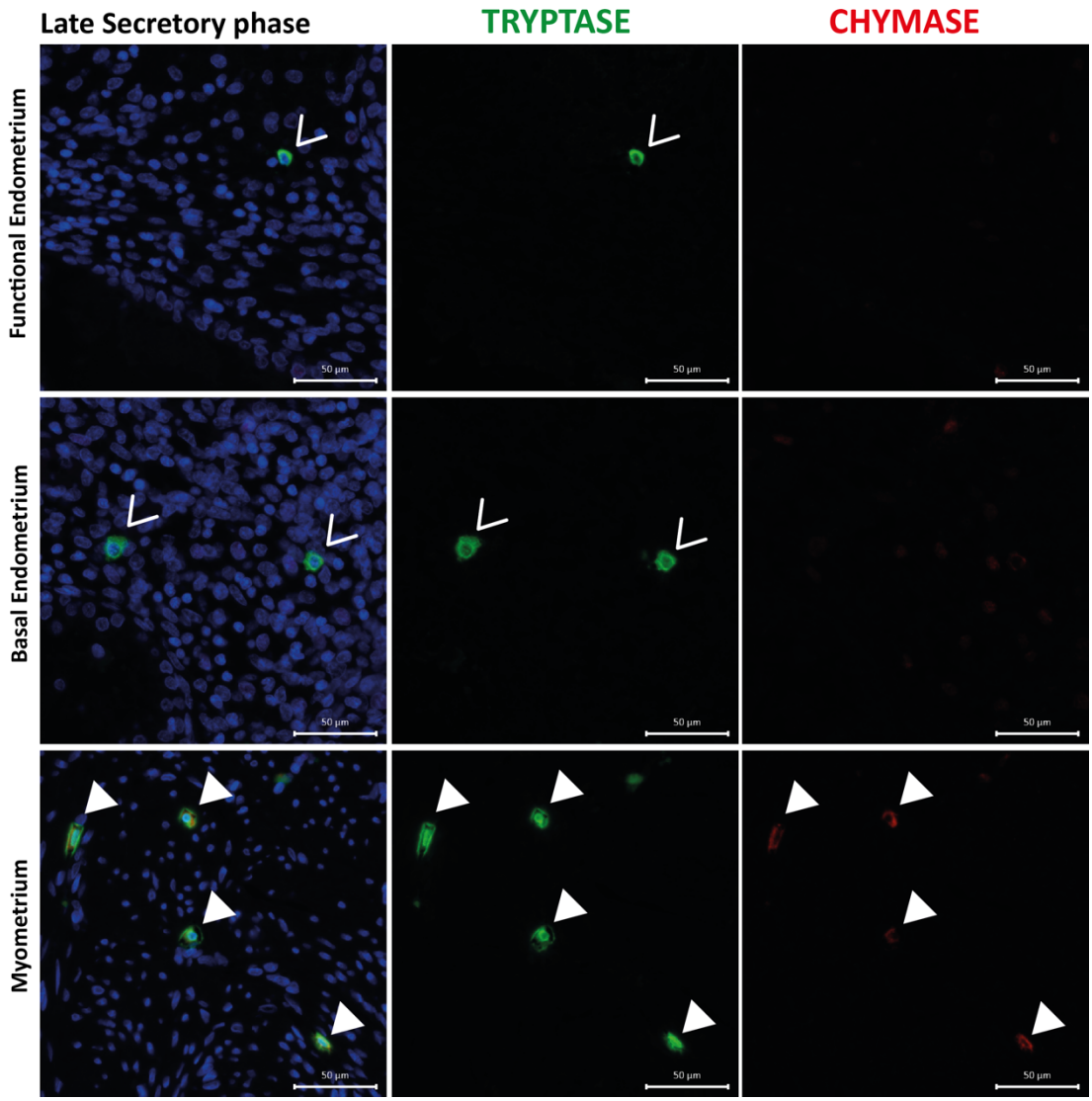

Figure 3-12 Mast cell subtypes and activation state during the late secretory phase.
During the late secretory phase, MCs are identified as tryptase single positive and weakly double positive (MC_{TC}). In both the endometrial layers MCs showed a strong tryptase only phenotype. MCs during the late secretory phase appeared to be activated only in the myometrial compartment, releasing tryptase and retaining chymase in the cytoplasm. (n=3) (White triangles: MC_{TC} cells; white Vs: MC_T cells).

membrane. Moreover, the MC phenotype appeared to be different when compared to the previous phases of the cycle, showing a low chymase content in MC_{TC} cells, no MC_C in the myometrium, and MC_T in the basal and functional compartments.

3.4.3.5 Menstrual phase

During the menstrual phase MCs in the basal endometrium had intense cytoplasmic staining for both tryptase and chymase (Figure 3-13). MCs were not detected in the shed functional layer of the endometrium. Interestingly, the numbers of MCs observed was higher when compared to the other phases of the cycle. The MC

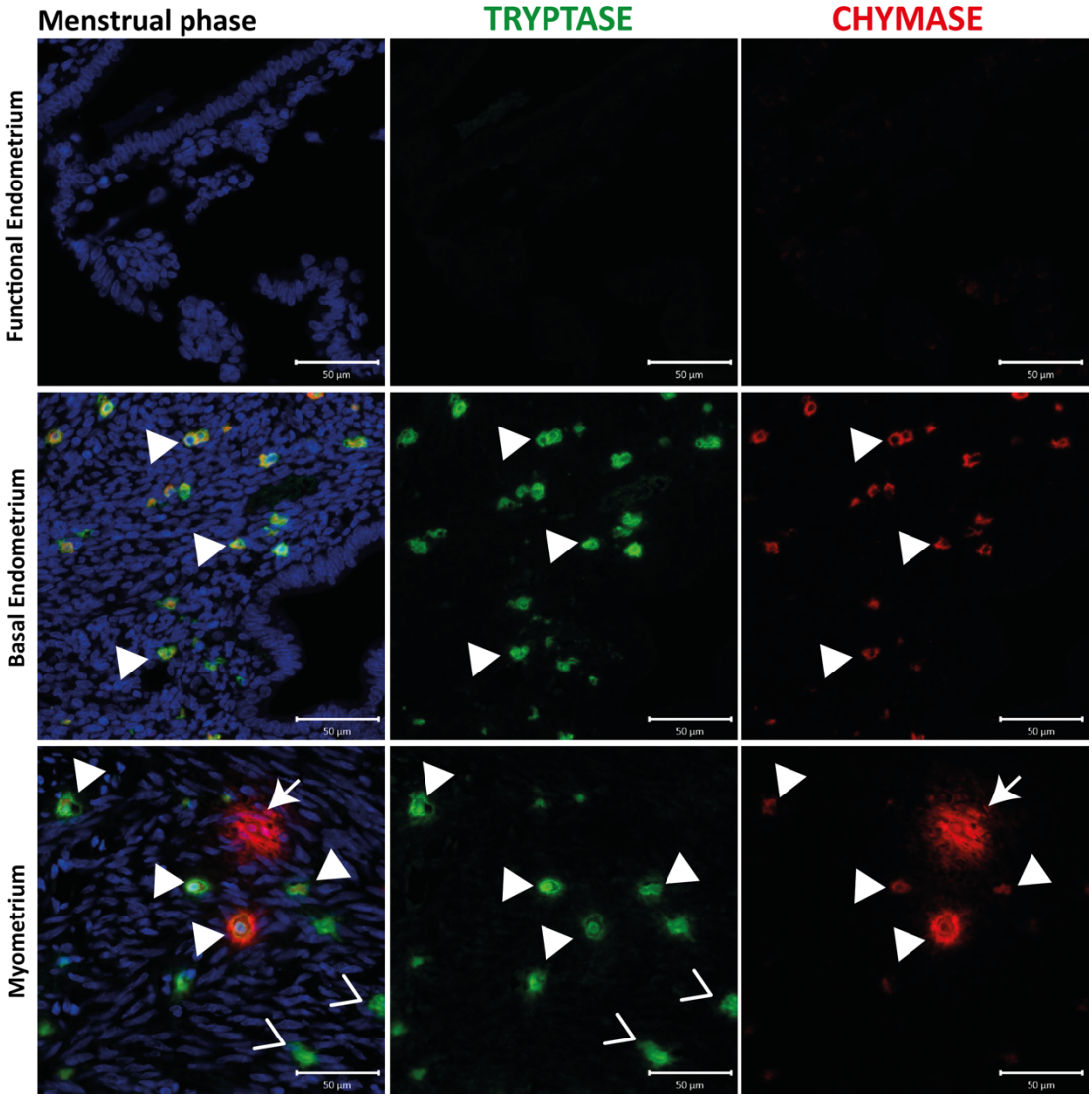

Figure 3-13 Mast cell subtypes and activation state during the menstrual phase.
Uterine mast cells appear to be tryptase and chymase double positive, in both myometrial and basal endometrial layers. MCs during the menstrual phase appeared to be activated only in myometrial compartment, releasing both tryptase and chymase from the cytoplasm. In the basal endometrium, MCs showed a steady state instead. (n=2) (White triangles: MC_{TC} cells; white Vs: MC_T cells; white arrows: MC_C cells).

phenotype within the different uterine layers during the menstrual phase, were MC_{TC} with high tryptase/low chymase, and MC_C only.

		Proliferative	Early Secretory	Mid Secretory	Late Secretory	Menstrual
MC subtypes	**Endometrium**	MC_T, MC_{TC}	MC_T, MC_{TC}	MC_T, MC_{TC}, MC_C	MC_T	MC_{TC}
		"resting"	"activated"	"resting"	"resting"	"resting"
	Myometrium	MC_{TC}, MC_C	MC_T, MC_{TC}, MC_C	MC_T, MC_{TC}	MC_{TC}	MC_{TC}, MC_C
		"resting"	"activated"	"activated"	"resting"	"activated"

Table 3-7 Summary table of uterine MC phenotypes and activation profile during the menstrual cycle

3.4.4 Immunolocalisation of oestrogen receptors shows ERβ is present in human uterine mast cells.

The available data on sex steroid hormone receptor expression in human MCs is based on a few studies in non-reproductive tissues (Zhao et al., 2001, Nicovani and Rudolph, 2002) and those using immortalized cell lines such as HMC-1 (Butterfield et al., 1988). These studies reported detection of progesterone receptor (PR), oestrogen receptor isoform α (ERα) but the oestrogen receptor isoform β (ERβ) was not identified (Zhao et al., 2001, Nicovani and Rudolph, 2002, Jensen et al., 2010)

The current investigation of the pattern of sex steroid hormone receptor expression in human uterine MCs started with validation of ERα immunoexpression in control tissues reported to contain ERα positive MCs, including skin, nasal polyps and tonsil (Figure 3-14). In contrast to previous findings, none of the positive control tissues were immunopositive for the oestrogen receptor isoform α (ERα) positive MCs, including samples from nasal polyps, tissue used by Zhao et al (2001). In the present study, first trimester decidua samples were also included as a positive control for ERα immunoreactivity (red staining in Figure 3-14).

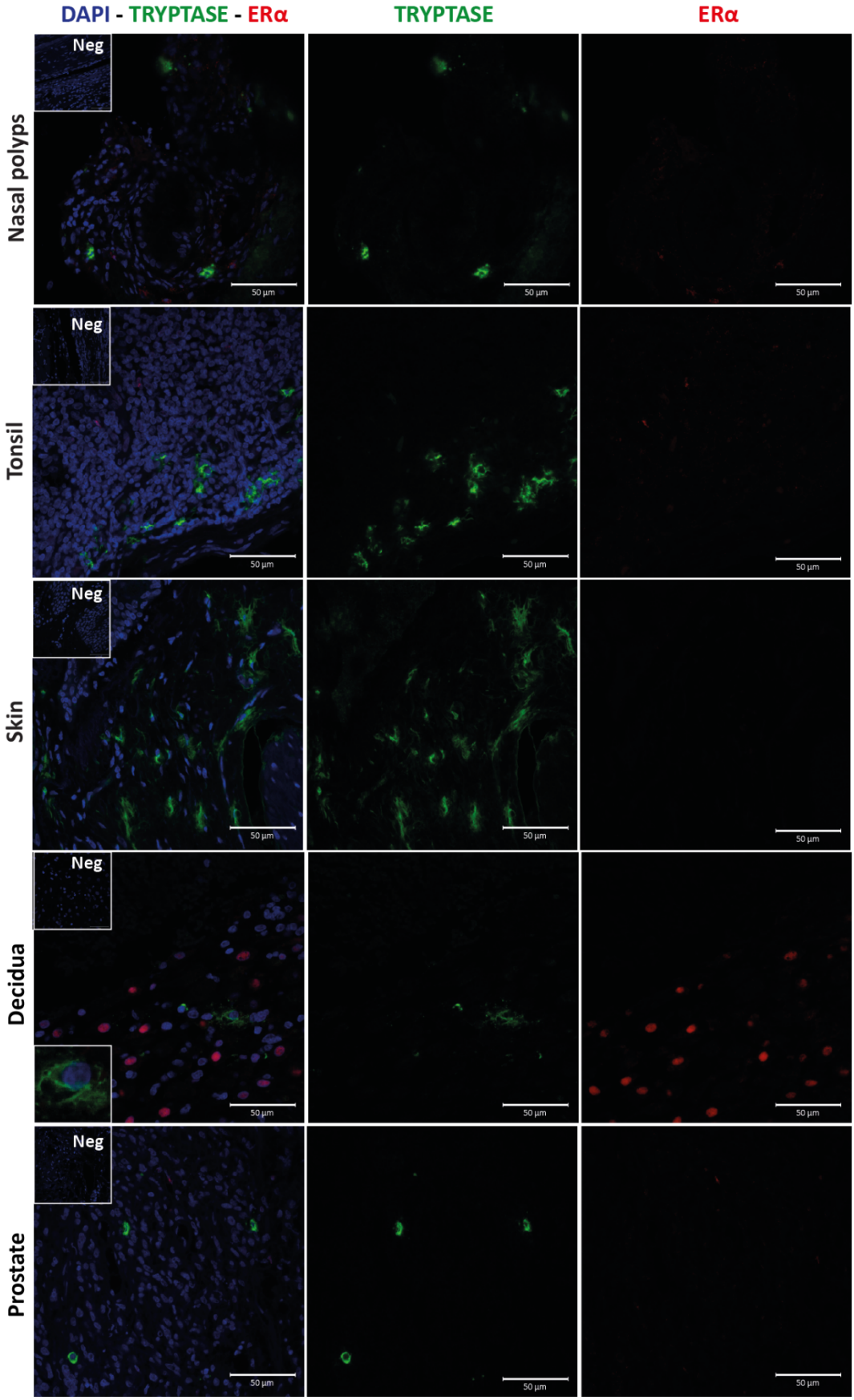

Figure 3-14 Mast cells and ERα expression in other human tissues.
Mast cells were found to be immunonegative for ERα (red staining; positive in decidual cells) in human nasal polyps, tonsil, skin and prostate. (Nasal polyps n=1, Tonsil n=1, Skin n=2, Decidua n=2 and prostate n=2.

ERα immunoexpression was detected in the human uterine biopsies. However, MCs present in the myometrium and basal layer of the endometrium were immunonegative for ERα in both proliferative and secretory phases of the menstrual cycle (Figure 3-15). In line with expectations, expression of ERα was detected in the nuclei of stromal and epithelial cells of the endometrium and in smooth muscle fibres in the uterine wall (Critchley et al., 2001).

In contrast to previous reports, the results presented in this study demonstrate that MCs in the human uterus are strongly immunopositive for ERβ (oestrogen receptor isoform β, Figure 3-16). Uterine MCs expressed ERβ independently of phase of the cycle and of location in the different compartments of the uterus. The expression pattern of ERβ in the stroma and epithelial compartments of the endometrium was consistent with our previous studies being higher during proliferative phase compared to the secretory phase (Critchley et al., 2002).

Zhao et al (2001) reported the immunoexpression of progesterone receptor in human MCs resident in the upper airways. MCs resident in the uterine environment were immunonegative for PR. This result was the same in both basal endometrium and myometrium, and was independent of the stage of the cycle (Figure 3-17).

Nuclear expression of ERβ in uterine leukocytes often parallels that of the glucocorticoid receptor (GR), uterine natural killer cells being one such example (Henderson et al., 2003). In the current study, nuclear GR was detected in MCs (Figure 3-18). MCs were immunopositive for GR regardless of their location, being GR positive in functional and basal endometrium and myometrium. In uterine cells, GR protein was sharply immunopositive in the endometrial stroma and smooth muscle fibres during the proliferative phase, but its expression was reduced during the progesterone-dominant secretory phase (Bamberger et al., 2001, Henderson et al., 2003). Figure 3-19 summarizes the unique steroid hormone receptor protein expression profile of uterine MCs. Notably, in this study double immunofluorescence demonstrated conclusively that mature uterine MCs do not contain ERα or PR as reported by Zhao et al (2001), but remarkably both ERβ and GR protein were localized to their nuclei.

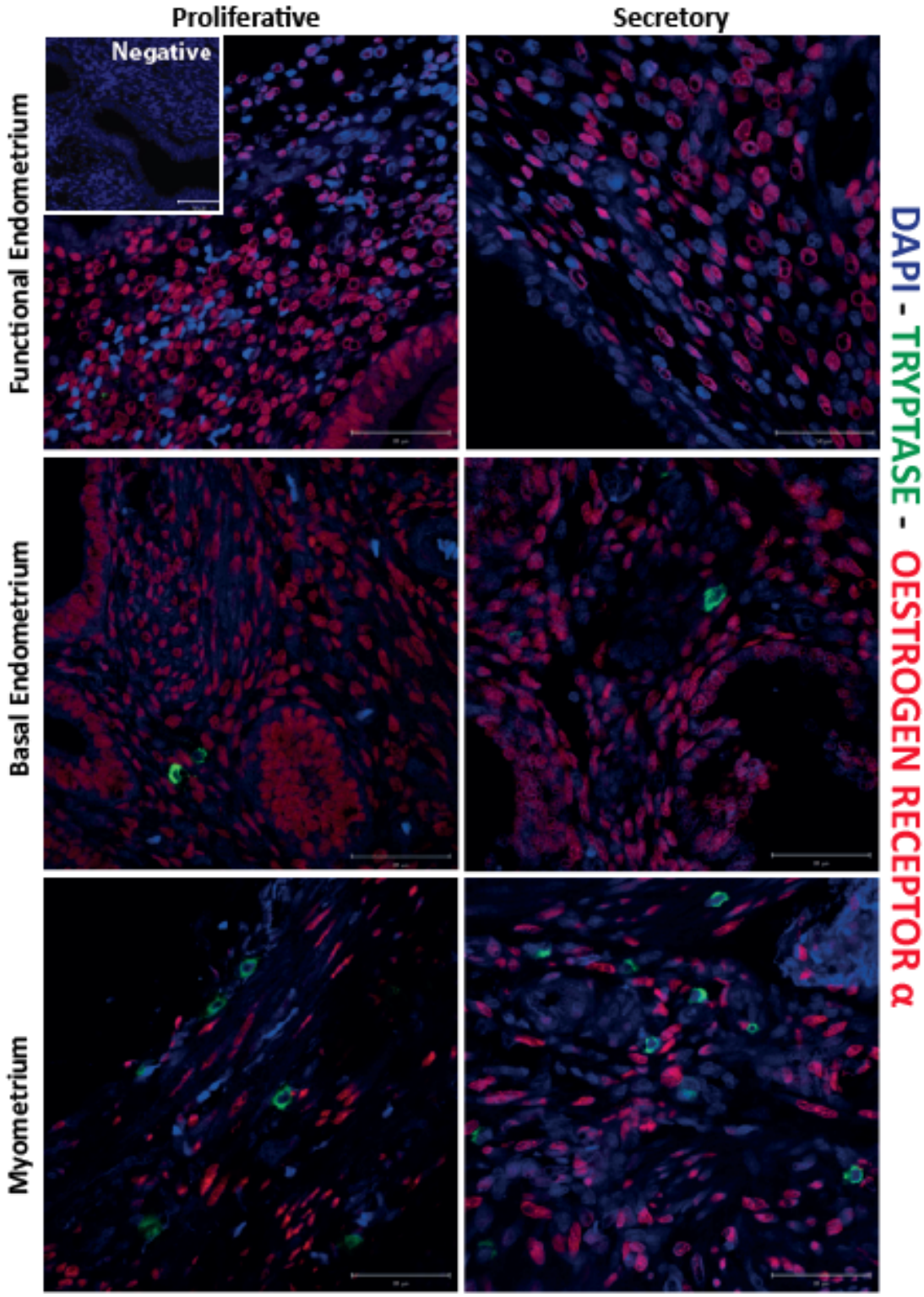

Figure 3-15 Uterine mast cells are immunonegative for ERα.
Double immunofluorescence showed no ERα expression (red staining) in the nuclei of uterine mast cells (green staining). Mast cells were noted to be immunonegative in all uterine layers and across the phases of the menstrual cycle. Myometrial, stromal and epithelial cells showed expression for ERα, as expected (Proliferative n=5, Secretory n=5).

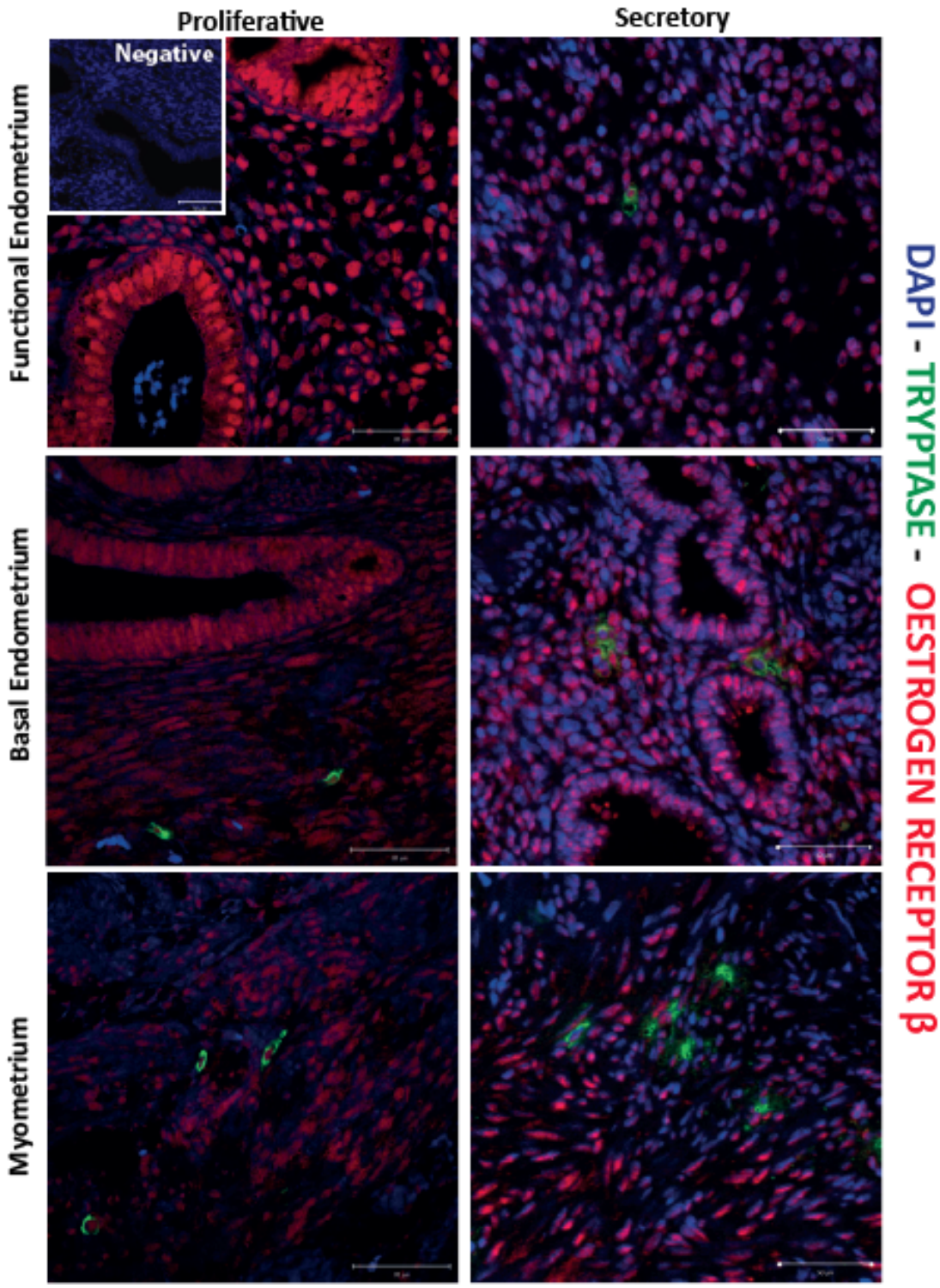

Figure 3-16 Uterine mast cells are immunopositive for ERβ.
Immunohistochemistry showed co-localization of ERβ in uterine mast cells. Nuclear expression of ERβ receptor (red staining) was detected in mast cells across the tissue structures of uterus, myometrium, basal and functional endometrium, and during both the proliferative and secretory phases of the menstrual cycle. (Proliferative n=5, Secretory n=5)

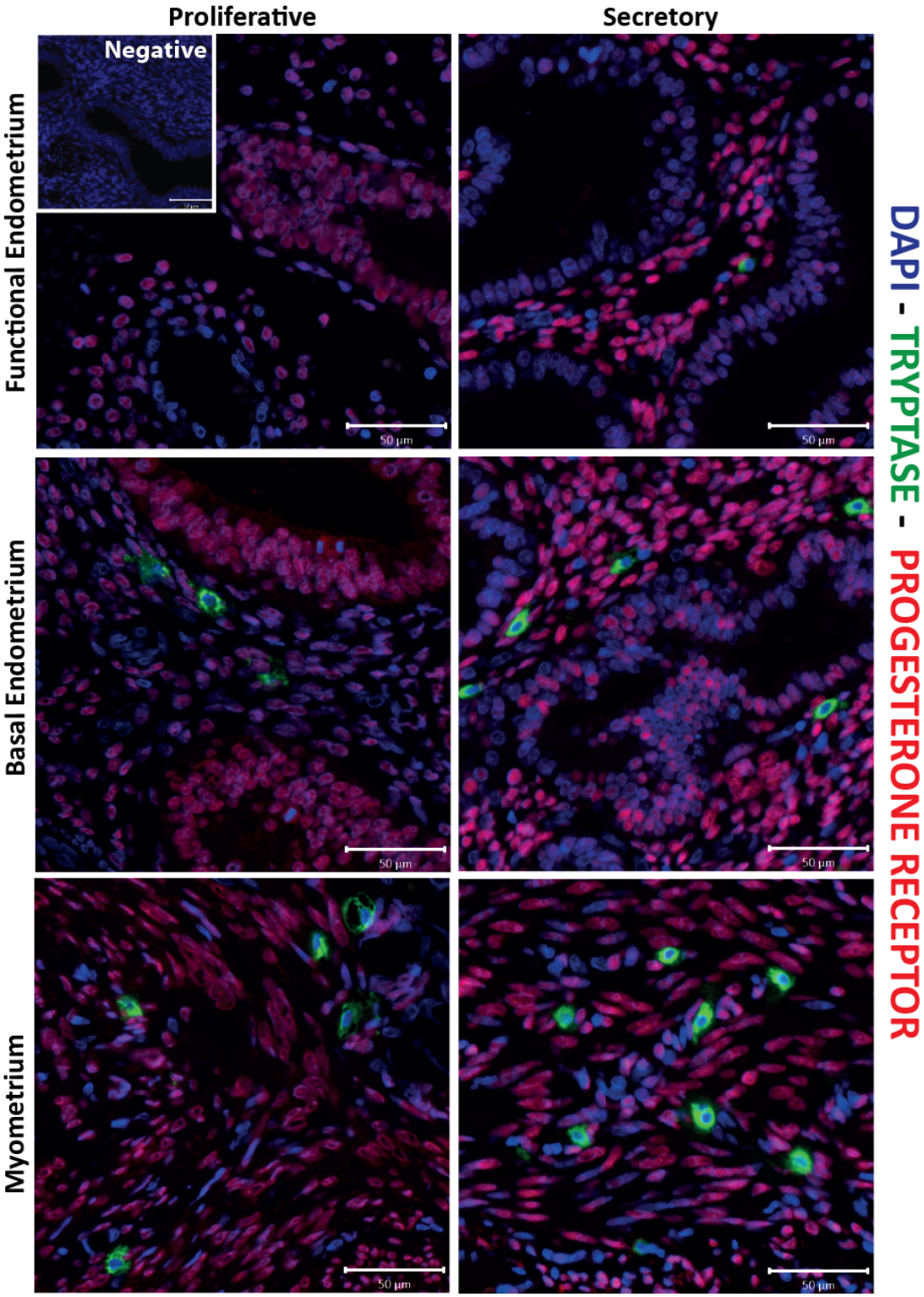

Figure 3-17 Mast cells and progesterone receptor expression in the human uterus.
Mast cells were demonstrated to be immunonegative for PR expression (red staining), across "full thickness" uterine sections and during both the proliferative and secretory phase. (Proliferative n=5, Secretory n=5).

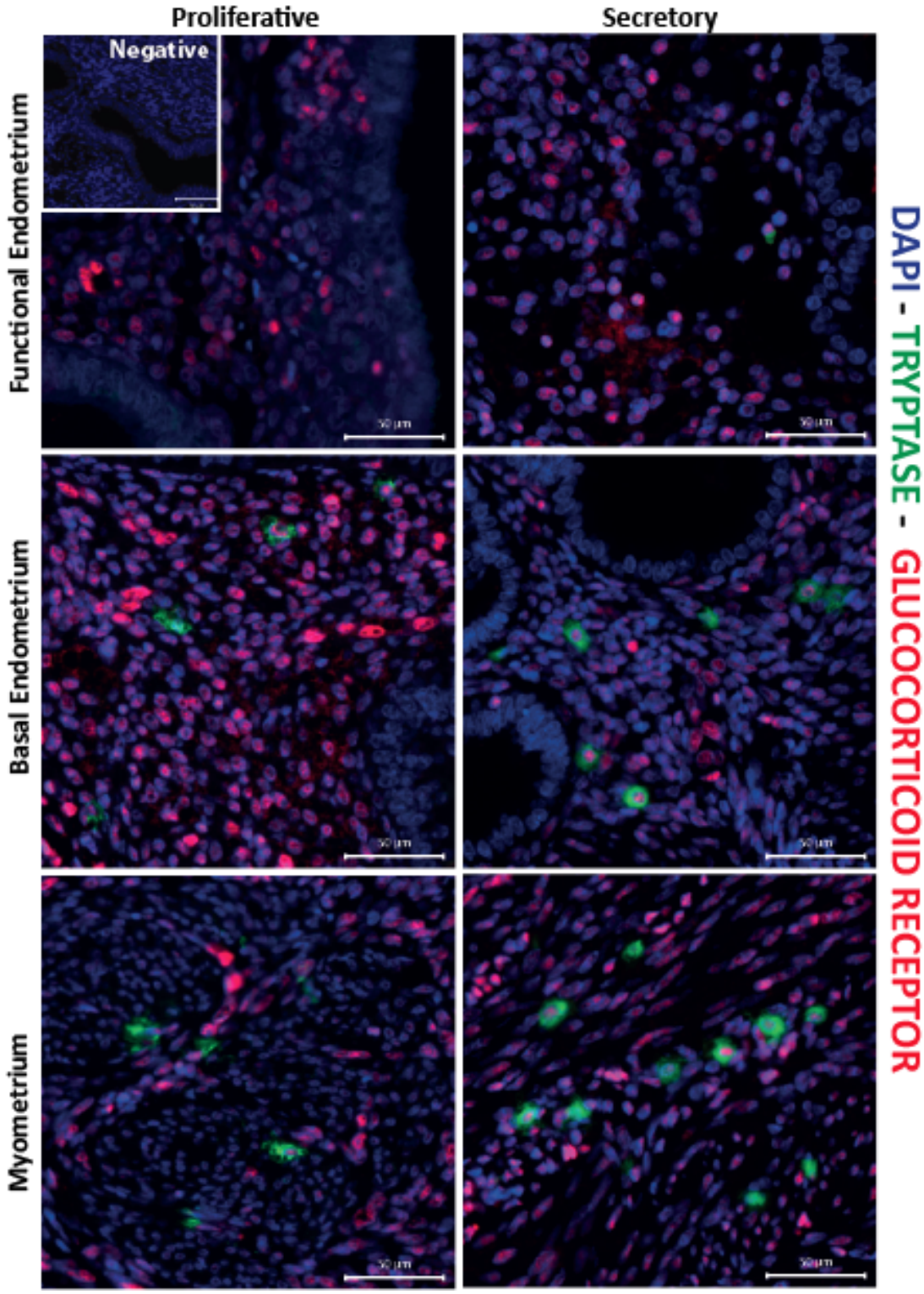

Figure 3-18 Mast cells express the nuclear activated form of glucocortoid receptor in the human uterus.

Uterine mast cells express the activated form of glucocorticoid receptor (GR; red staining) during the proliferative and secretory phase, throughout the myometrium, functional and basal endometrial and layers. (Proliferative n=5, Secretory n=5).

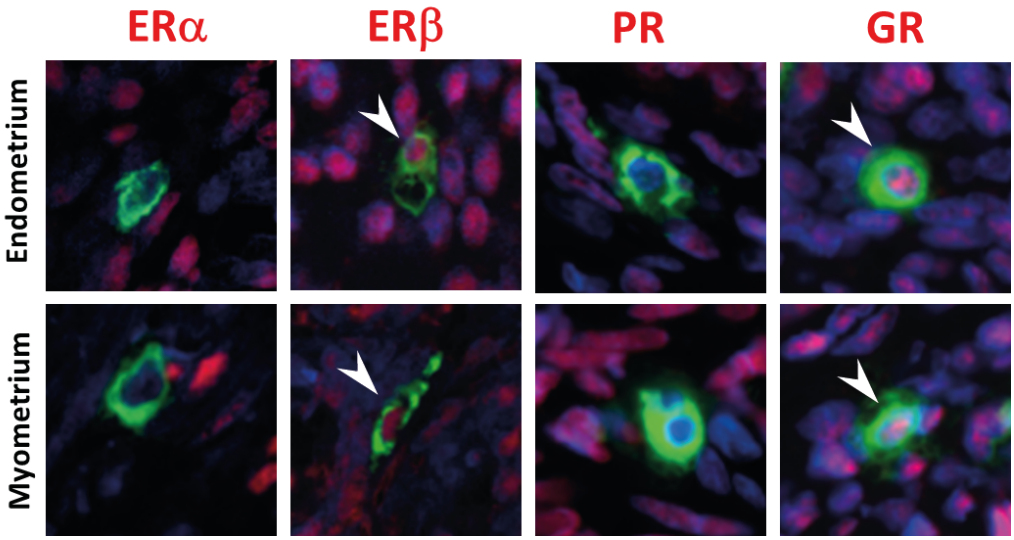

Figure 3-19 Representative steroid receptor expression panel in human uterine mast cells
Uterine MCs are immunopositive for ERβ and GR, and immunonegative foe ERα and PR. **RED staining: ERα, ERβ, PR and GR, GREEN staining: tryptase.**

3.5 Discussion

While other uterine leukocytes have been extensively studied, only a few studies have investigated the nature of the mature mast cells resident in the human uterus. This study investigates both the numbers of mast cells in the uterus (endometrium and myometrium) as well as their phenotype in the specific microenvironment of the human uterus.

The cellular and extracellular composition of human endometrium undergoes extensive remodelling during every phase of the menstrual cycle. This dynamic remodelling is orchestrated by the ovarian steroid hormones, oestrogen and progesterone, and their fluctuating serum levels. There is compelling evidence that the leukocyte population contributes to the tissue adaptation across the phases of the cycle (Evans and Salamonsen, 2012a). Notably, both the total number of immune cells and the relative numbers of each immune cell type varies according to the different stages of the menstrual cycle. The lympho-myeloid cell types in the endometrium include eosinophils, macrophages, neutrophils, uterine natural killer and mast cells. Whereas the influx of eosinophils and neutrophils is apparent during the pre-menstrual and late secretory phases, macrophages and natural killers increase in number immediately after ovulation and during the decidualization process (Kamat and Isaacson, 1987,

Kammerer et al., 2004). Other cells like mast cells are reported to be consistent in number throughout the menstrual cycle (Salamonsen et al., 2002).

The current investigation, undertaken 15 years after the last published study of uterine mast cells (Sivridis et al., 2001), confirmed with modern technologies that mature mast cells are resident in the human uterus. Firstly, confirmation of the uterine MCs was accomplished with analysis of the expression of mRNA's encoded by proteases in endometrial tissue biopsies. This is the first time, mRNAs for mast cell specific proteases have been quantified in endometrial tissue homogenates. The concentrations of tryptase and chymase mRNAs remained unchanged in the endometrium during the different phases of the menstrual cycle. I speculate that these results may be influenced by the life span of tissue resident mast cells. For example, *in vivo* studies have reported that mast cells have a lifespan of weeks to months in tissues (Padawer, 1974, Kiernan, 1979). As tryptase and chymase are constitutively expressed proteases in mast cell granules (Pejler et al., 2007), it is not unexpected that expression of their mRNAs might remain unchanged during their long life span.

Furthermore, the potential downstream effects of mast cell mediators released during activation were also investigated by measuring the concentration of mRNAs encoded by histamine receptor 1 (*HR1*) and protease-activated receptor 2 (*PAR-2*), which are activated by histamine and tryptase respectively. Histamine has often been reported to be part of the angiogenetic machinery (Lu et al., 2013). Angiogenesis is the formation of new blood vessels, and occurs in the endometrium during tissue remodelling in the proliferative phase and during formation of spiral arterioles in early stages of the secretory phase (Rogers, 1996, Demir et al., 2010). The pro-angiogenetic action of histamine has been reported to work through HR1 and HR2 receptor-mediated synthesis of vascular endothelial growth factor (VEGF) (Sorbo et al., 1994, Rizzo and Defouw, 1996). Indeed, several studies have showed that VEGF is involved in regulation of endothelial cell growth, angiogenesis and permeability in the human endometrium (Sugino et al., 2002, Punyadeera et al., 2006).

PAR receptors are G protein-coupled receptors (GPCRs) that are uniquely activated by proteolysis (Soh et al., 2010). The isoform 2 of PAR receptor is predominately cleaved by mast cell tryptase and trypsin (Compton et al., 2001). PAR-

2 is mainly expressed in vascular, epithelial, fibroblastic and immune cells. Its activation has been reported to be important in the processes of haemostasis and inflammation (Coughlin, 2000). PAR-2 activation regulates ion transport in epithelial cells, by increasing levels of intracellular calcium (Kunzelmann et al., 2002). There is evidence that PAR-2 activation mediates a proliferative response in human endothelial cells (Mirza et al., 1996) and it influences prostaglandin synthesis by upregulating synthesis of the cyclooxygenase 2 enzyme (COX-2) in fibroblasts (Seymour et al., 2005, Zhang et al., 2012). Furthermore, cleavage of the N-terminal domain of PAR-2 can trigger an immune response indirectly by the release of the chemoattractant interleukin-8 from epithelial and endothelial cells (Asokananthan et al., 2002), and directly by enhancing motility of neutrophils (Shpacovitch et al., 2004) and activating eosinophils (Miike et al., 2001).

mRNAs encoded by *HR1* and *PAR-2* were detected at very high concentrations in human endometrium when compared to control tissues, such as tonsil. *HR1* mRNA remained constant throughout the different phases of the menstrual cycle, consistent with a possible role for histamine during tissue remodelling and angiogenesis in the human endometrium. *PAR-2* appeared to be upregulated, in a non-significant manner, during the secretory phase compared to proliferative phase, a time that is characterized by epithelial growth, decidualization and immune cell influx. PAR-2 protein was also investigated, and it was demonstrated that this protease-activated receptor was present in the human endometrium, in both stromal and epithelial compartments. This finding would be consistent with potential activation of PAR-2 in endometrial and epithelial cells as described by Hirota et al. (2005).

Quantification of mast cells conducted during this study has revealed new evidence that their phenotype in both the basal endometrium and myometrium was predominately one of co-expression of tryptase/chymase. Mast cells were rarely detected in the functional endometrium and in this compartment they not only appeared to be of the MC_T phenotype but the tryptase was detected within the cytoplasmic granules and did not appear to be released in the surrounding tissue. Two methods were used, first DAB staining for tryptase and chymase was conducted separately on serial sections, repeating the study reported by Jeziorska et al. (1995)

and second, double immunofluorescent staining was undertaken allowing simultaneous detection of both proteases in the same cell section, which coupled with the tyramide amplification system is demonstrated to have 1000-fold signal amplification compared to DAB detection.

Detailed analysis of the uterine MC's phenotype in multiple samples for each phase of the cycle, has revealed the presence of a rare novel third subtype not previously described in the endometrium: the chymase-only positive mast cell (MC_C). Traditionally, human mast cells were classified according to their proteolytic granules based on the expression of tryptase and/or chymase (Irani et al., 1986). Weidner et al. (1993) were the first scientists to report the existence of chymase positive/tryptase negative (MC_C) mast cells. MC_C were subsequently detected by immunofluorescence in the airway and gastrointestinal tract, reported as 12% in human bronchi and 16.8% bowel submucosa of total MC number. The presence of a MC_C population was also investigated in cases of organ rejection, such as following a kidney transplant (Yamada et al., 2001). The current study provides further evidence for the presence of three different subtypes of mast cells. In this study the phenotype of uterine MCs was extensively studied and it was evident that the different phenotypes were both stage and tissue compartment specific. For example, MC_C were detectable only in the myometrium and the basal endometrium during the mid secretory phase. This finding would appear to be consistent with the concept of phenotypical plasticity that has been advanced by Galli et al (2011). The current findings would suggest that uterine mast cells adopt a chymase-positive phenotype facilitating stage specific impacts which may include the formation of endometrial arterioles from arcuate arteries in the myometrium (Farrer-Brown et al., 1970).

The activation state of uterine MCs was further explored during the present study. Generally, uterine MCs are reported to degranulate during the secretory phase at a time when the endometrial tissue is in an oedematous state. This observation was based on detection of extracellular tryptase during oedema and weak intracellular tryptase staining detectable during the proliferative phase (Jeziorska et al., 1995). In this study, endometrial activation and the degranulation of MCs was documented during the early and mid-secretory stages of normal uterine tissue with detection of

tryptase and chymase in the extracellular matrix. A "recovery" state, characterised by weak immunostaining, was observed in tissue collected from patients during the proliferative, late secretory and menstrual stages. Within the myometrial compartment MCs were in a "resting" state only during the proliferative phase. Interestingly, in the myometrium MCs were activated during both secretory and menstrual phases, suggesting a potential role for MCs in regulation of arteriole sprouting at early/mid secretory phases and smooth muscle contraction during menses. These findings conclusively demonstrated that MC behaviour is cycle phase dependent and prompted, further investigation as to the possible impact of sex hormones on the degranulation process.

Female sex hormones have long been suspected to affect the behaviour/action of mast cells, in part because many MC-related conditions have a higher prevalence in women than in men. For example, the incidence of asthma is greater in women during early to mid-adulthood, it worsens perimenstrually and during menopause it is aggravated by hormonal replacement therapies (Bonds and Midoro-Horiuti, 2013). Furthermore, there is also some evidence for the expression of sex steroid hormone receptors in human mast cells (Zhao et al., 2001, Nicovani and Rudolph, 2002). However, the current study is to my knowledge the first investigation to elucidate the steroid hormone receptor expression profile of mast cells in the human uterus. Based on current literature, the results obtained were unexpected as dual immunofluorescence revealed that uterine MC did not contain detectable oestrogen receptor α (ERα) or progesterone receptor (PR). Notably, the uterine MCs were immunopositive for oestrogen receptor β (ERβ) and this expression was independent of the phase of the menstrual cycle. Interestingly, we have previously documented that other uterine resident immune cells, such as uterine natural killer cells (uNKs) and macrophages have a similar expression pattern, being immunopositive for ERβ and immunonegative for ERα (Henderson et al., 2003, Thiruchelvam et al., 2016).

Several studies have reported that glucocorticoids may have an indirect anti-inflammatory impact on mast cells, with the postulated mechanism being a reduction of stem cell factor production by fibroblasts (Da Silva et al., 2002). Alternatively, they may also have direct impacts by reducing IgE binding to the FcεRI receptors thereby

downregulating the expression of these receptors on the cell membrane of the mast cells and inhibiting MC degranulation *in vitro* (Finotto et al., 1997, Yamaguchi et al., 2001, Zhou et al., 2008). Prior to the current study, the only report of expression of glucocorticoid receptor (GR) expression in human mast cells was from Oppong et al. (2014). In their study, they localized GR to the plasma membrane in RBL-2H3 mast cells. The current study is the first to demonstrate that uterine mast cells are immunopositive for nuclear GR. A glucocorticoid rich environment would favour activation of GR, with shuttling of ligand activated receptor from the cytoplasm towards the nucleus (Phuc Le et al., 2005). This observation is due to high uterine endometrial levels of glucocorticoids. This finding is consistent with local biosynthesis of cortisol and expression 11-β hydroxysteroid dehydrogenase enzymes (11β-HSDs) within the uterus (McDonald and Henderson, 2006, Gibson et al., 2013).

Although, other authors have demonstrated a direct effect of female sex hormones on mast cell behaviour, activation and migration, in those studies they cited activation of MCs via ERα and PR (Zaitsu et al., 2007, Jensen et al., 2010). In contrast, the current data suggests that the impact of oestrogens on uterine mast cells may be mediated via ERβ. This speculation is also supported by the activation of uterine mast cells during the secretory phase. This is a time in the menstrual cycle when intracrine biosynthesis of oestradiol has been shown to activate ERβ positive uNKs (Gibson et al., 2015).

In summary, the studies described in this chapter confirm that mast cells are a component of the leukocyte population of the human uterus, and are most abundant in the myometrial and basal compartments. The data show that uterine MCs predominantly belong to the classic mast cell subtypes: tryptase positive/chymase negative (MC_T) and tryptase/chymase positive (MC_{TC}), but a rare third subtype was also identified in the uterine population: the tryptase negative/chymase positive uterine MCs (MC_C). Mast cell activation/degranulation was menstrual cycle stage dependent, and their environment-specific phenotype revealed immunoexpression of ERβ and GR, suggesting they are a target cells for both oestrogens and glucocorticoids.

The limitation of the current study is in the limited availability of human samples which has led to a lack of statistical power in the results. Therefore, further investigations would be needed to confirm the findings of this chapter.

Chapter 4 Characterization of phenotype of mast cells in women with pain and endometriosis

4.1 Introduction

Endometriosis is a chronic incurable condition defined as the presence of endometrial tissue ("lesions"), composed of stromal and epithelial cells, outside the uterine cavity. Prevalent locations for endometriotic lesions are: the pelvic peritoneum, ovaries and recto-vaginal septum (Burney and Giudice, 2012). The aetiology and pathogenesis of endometriosis are still not clear, although one theory initially proposed by Sampson (1927), states that endometriotic lesions may originate from retrograde menstruation and ectopic endometrial tissue adhesion and invasion after the reflux of endometrial cells via the Fallopian tubes.

Endometriosis is most prevalent in women of reproductive age (affects one in ten women) and regresses after menopause, after circulating sex hormones decrease (Cramer and Missmer, 2002). The symptoms of endometriosis can be extremely debilitating including chronic pelvic pain, dysmenorrhea (painful menstruation), pain at ovulation, dyspareunia, irregular and/or heavy menstrual bleeding, fatigue and infertility (Giudice, 2010). Endometriosis, due to its presentation and multifactorial nature, is often under-diagnosed and is associated with an average of 7-year delay from the onset of symptoms to a definitive diagnosis (Simoens et al., 2012).

Factors considered to be key regulators of the establishment and development of endometriotic lesions include: a) increased adhesion and invasiveness of shed endometrial cells in the peritoneal cavity; b) alterations of hormone levels in the peritoneum, with the lesion due to local biosynthesis of oestrogen; c) "progesterone resistance" resulting in endometrial dysfunction; and d) an aberrant inflammatory environment with increased secretion of growth factors and cytokines that triggers the influx and activation of immune cells (eg. macrophages, uterine natural killer cells, and mast cells (MCs) (Signorile and Baldi, 2010, Burney and Giudice, 2012, Taylor et al., 2012, Kobayashi et al., 2014).

Development of endometriotic lesions is also sustained by neuro-angiogenic processes which enable nutrient supply and innervation of the lesions (Wang et al., 2009). Chronic pelvic pain (CPP) is a debilitating symptom of several disorders, including endometriosis, irritable bowel syndrome, interstitial cystitis and musculoskeletal and neurological diseases (Triolo et al., 2013). Endometriosis-associated pelvic pain is thought to be due to abnormal inflammation, oxidative stress, angiogenic, neurovascular, or neuropathic mechanisms in the lesion/peritoneum environment (Kobayashi et al., 2014). Different pathways are thought to be the cause of CPP experienced by women with endometriosis: one of these is that the endometriotic lesion could cause a compression or infiltration of nerves (Anaf et al., 2000, Morotti et al., 2014). On the other hand, Triolo et al. (2013) suggested that a rise in perception of CPP could be a result of an increase in the release of nerve growth factor (NGF) and substance P in the endometriotic lesion area, and this may have an impact on subsequent nerve growth potentially increasing the links between new pain afferents and the central nervous system.

Mast cells are immune cells of the myeloid lineage, derived from pluripotent precursors of the bone marrow (Kirshenbaum et al., 1999, Nielsen and McNagny, 2009). Mature MCs may be found in most tissues of the body, but they are typically most abundant at sites of host-environment defence, such as the skin and other mucosal tissue; bronchi and intestine for example (Metcalfe et al., 1997). The most distinguishing morphological MC characteristic is their high content secretory granules, which occupy the majority of the cytoplasm of mature cells (Padawer, 1974). MCs are classified based on their granule content of serine proteases, such as tryptase and chymase (Metcalfe et al., 1997).

MC secretory granules are filled with preformed compounds including: serine protease such as tryptase and chymase; biogenic amines such as histamine, serotonin and dopamine; and other cytokines and growth factors such as tumour necrosis factor (TNF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) (Wernersson and Pejler, 2014). Upon stimulation, MCs synthetize and store within their granules eicosanoids, such as prostaglandins D_2 (PGD_2) and E_2 (PGE_2),

leukotriene C_4 (LTC_4), as well as interleukins (IL-3, IL-5, IL-8) and chemokines, such as CCL3 (Abraham and St. John, 2010).

Detailed studies during Chapter 3, demonstrated that the human endometrium contains MCs with a range of phenotypes, predominantly tryptase positive in the functional layer. Uterine MC activation was shown to be menstrual cycle stage dependent. It was further elucidated in chapter 3 MC ability to respond to steroids, due to their immunoexpression of ERβ and GR, suggesting they are a target cells for both oestrogens and glucocorticoids.

The activation of MCs and subsequent release of the granule compounds from MC granules significantly modulates many aspects of physiological and pathological conditions. For example, mast cells may regulate vasodilation, vascular homeostasis, immune responses and angiogenesis.

MCs are also known as key players in both physiological and pathological pain pathways and in the pathology of diseases. Interestingly, MCs are often found close to primary nociceptive neurons and can participate in neuro-immune synapse signalling (Dropp, 1972, Stead, 1989). MCs and sensory neurons are involved in an interactive activation loop: MC mediators sensitize sensory neurons, which further activate the surrounding MCs by releasing neuropeptides such as NGF, histamine and substance P (Rosa and Fantozzi, 2013).

Recently, a few studies have described an increase in the number of activated mast cells in peritoneal endometriosis, indicating a potential function for mast cells and their mediators in the onset of fibrosis in lesions (Kempuraj et al., 2004, Sugamata et al., 2005, Kirchhoff et al., 2012). Additionally, Anaf et al. (2006) showed that deep infiltrating lesions were associated with a higher number of activated MCs located in intimate proximity with the sensory nerves.

Taken together these findings suggest that activated MCs might play a key role in endometriosis, a condition where immune, neural, and apparently endocrine components are altered.

4.1.1 Summary

Endometriosis is complex multifactorial gynaecological condition, which affects up to 10% of women of a reproductive age worldwide (Simoens et al., 2012). It is defined as the growth of endometrial tissue outside the uterine cavity, primarily in the peritoneal cavity and ovaries (Giudice and Kao, 2004). Its most common symptom reported, is chronic pelvic pain, which determines endometriosis as a condition with social impact (Simoens et al., 2012).

The peritoneal environment of women affected by endometriosis has an increased inflammatory profile with regards to the numbers of immune cells recruited to the tissue and pro-inflammatory mediator release (Signorile and Baldi, 2010). Different immune cell types have been intensively studied in endometriosis patients (Burney and Giudice, 2012), but very few studies have investigated the role of mast cells in the pathophysiology of endometriosis and its chronic pelvic pain symptom.

Taken together, the hypothesis of this study is that MCs are involved in the establishment and/or maintenance of endometriosis and chronic pelvic pain, through their sex hormone dependent degranulation and their role in neuro-inflammation.

4.2 Aims of the Chapter

1. To explore the phenotype of mast cells in the eutopic endometrium and peritoneum of women with chronic pelvic pain and compare it to unaffected women.
2. To investigate mast cells and their mediators in the peritoneal fluid of women suffering with chronic pelvic pain and/or endometriosis

4.3 Methods

4.3.1 Human tissue resources

Human tissue was obtained from women undergoing laparoscopy for non-malignant gynaecological conditions or sterilisation procedures. All patients were within the 18-49-year age range and had menstrual cycles between 24-35 days in length (Table 4-1). Local ethical committee approval was granted and written informed patient consent was obtained prior to tissue collection. Local ethical approval was held by Professor A.W. Horne (11/AL/0376).

Endometrium from the functional layer (eutopic endometrium) was collected by using a suction "pipelle" endometrial sampling device. The tissue biopsies collected during laparoscopy were "peritoneum prone", from the recto-vaginal pocket (pouch of Douglas, "prone" site for endometriotic lesion formation) and "peritoneum distant" from the frontal abdominal peritoneal wall ("distant" site from endometriotic lesion formation), whether present, ectopic lesions were also collected. Peritoneal fluid was aspirated from the pouch of Douglas (Figure 4-2).

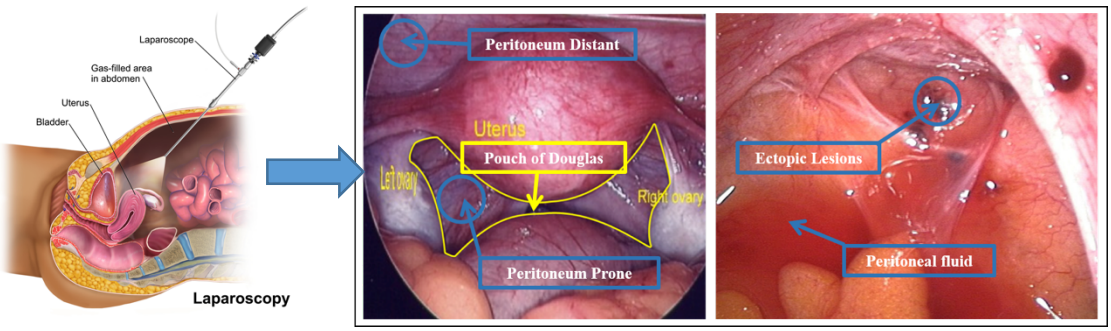

Figure 4-1 Laparoscopic view of the peritoneal cavityand sites for tissue biopsies collection. Images adapted from Hic and nunc (2011) and Blaus (2014)

Patients attending a chronic pelvic pain clinic were classified according the American Fertility Society's guidelines (Canis et al., 1997). Patients were subdivided as follows: Grade 0 - women affected by chronic pelvic pain without endometriosis found during diagnostic laparoscopy; Grade I - women with chronic pain and with minimal endometriosis; Grade II - women with chronic pain and mild endometriosis; Grade III - women with chronic pain and moderate endometriosis and Grade IV - women with chronic pain and severe endometriosis. This control group was denominated as the "no pain" control group, because some of them were suffering by

asymptomatic endometriosis. Full details of patient characteristics including menstrual cycle stage (confirmed by histo-pathology) and whether they were receiving hormonal therapy as well as tissue resources are detailed in Table 4-1 and Table 4-2.

Unique No	AFS Grade	Exogenous hormones	H&E Stage	Age	Average Menstrual cycle length	Endometriosis	Pain	Sample Application
3454	0	NO	NK	36	28	✗	✓	ELISA/FLOW
3442	0	NO	NK	30	NK	✗	✓	RNA/ELISA/FLOW
3421	0	NO	NK	41	30	✗	✓	ELISA
3378	0	NO	NK	29	NK	✗	✓	ELISA/FLOW
3300	0	NO	NK	21	NK	✗	✓	ELISA
3180	0	NO	Proliferative	40	28	✗	✓	ELISA
3234	0	NO	Proliferative	18	28	✗	✓	IF/ELISA
3246	0	NO	Proliferative	36	31	✗	✓	RNA
3282	0	NO	Proliferative	45	NK	✗	✓	IF
3298	0	NO	Proliferative	47	25	✗	✓	IF/ELISA
3319	0	NO	Proliferative	38	30	✗	✓	ELISA
3484	0	NO	Proliferative		NK		✓	ELISA
3491	0	NO	Proliferative	49	24-31	✗	✗	RNA/IF/ELISA/ FLOW
3185	0	NO	Early Secretory	32	35	✗	✓	ELISA
3190	0	NO	Early Secretory	29	21	✗	✓	ELISA
3205	0	NO	Early Secretory	39	28	✗	✓	IF/ELISA
3241	0	NO	Early Secretory	32	30	✗	✓	IF/ELISA
3295	0	NO	Early Secretory	30	28	✗	✓	ELISA
3438	0	NO	Early Secretory	32	35	✗	✓	RNA/ELISA/FLOW
3472	0	NO	Early Secretory	26	32-38		✓	FLOW
3497	0	NO	Early Secretory	34	<24	✗	✗	RNA/IF/ELISA/ FLOW
3247	0	NO	Mid Secretory	36	35	✗	✓	RNA
3290	0	NO	Mid Secretory	37	29	✗	✓	ELISA
3343	0	NO	Mid Secretory	39	21	✗	✓	ELISA
3443	0	NO	Mid Secretory	38	28	✗	✓	RNA/ELISA
3469	0	NO	Mid Secretory		NK		✓	ELISA/FLOW
3203	0	NO	Late Secretory	36	28	✗	✓	IF
3272	0	NO	Late Secretory	28	31	✗	✓	RNA/ELISA
3384	0	NO	Late Secretory	25	24	✗	✓	RNA/ELISA/FLOW
3388	0	NO	Late Secretory	36	NK	✗	✓	RNA/ELISA/FLOW
3427	0	NO	Late Secretory	27	28	✗	✓	RNA
3450	0	NO	Late Secretory	31	30	✗	✓	ELISA/FLOW

3245	0	NO	Menstrual	28	28	✗	✓	IF/ELISA
3334	0	NO	Menstrual	32	28	✗	✓	IF
3426	I	NO	NK	23	NK	✓	✓	ELISA/FLOW
3376	I	NO	NK	33	32	✓	✓	ELISA/FLOW
3192	I	NO	NK	28	26	✓	✓	ELISA
3294	I	NO	Proliferative	30	31	✓	✓	IF
3320	I	NO	Proliferative	40	23	✓	✓	IF/ELISA
3355	I	NO	Proliferative	28	32	✓	✓	ELISA/FLOW
3374	I	NO	Proliferative	35	28	✓	✓	FLOW
3398	I	NO	Proliferative	23	30	✓	✓	RNA/ELISA/FLOW
3404	I	NO	Proliferative	25	NK	✓	✓	RNA
3448	I	NO	Proliferative	32	NK	✓	✓	ELISA/FLOW
3459	I	NO	Proliferative	45	NK	✓	✓	ELISA
3494	I	NO	Proliferative	31	24-31	✗	✗	RNA/IF/ELISA/ FLOW
3221	I	NO	Early Secretory	30	30	✗	✓	IF/ELISA
3232	I	NO	Mid Secretory	26	28	✓	✓	IF/ELISA
3248	I	NO	Mid Secretory	43	NK	✓	✓	ELISA
3254	I	NO	Mid Secretory	28	32	✓	✓	ELISA
3306	I	NO	Mid Secretory	36	NK	✓	✓	ELISA
3307	I	NO	Mid Secretory	40	28	✓	✓	IF/ELISA
3446	I	NO	Mid Secretory	39	NK	✓	✓	RNA/ELISA
3466	I	NO	Mid Secretory	35	32-38		✓	FLOW
3228	I	NO	Late Secretory	40	28	✓	✓	ELISA
3451	I	NO	Late Secretory	26	21	✓	✓	RNA
3278	II	NO	Proliferative	25	31	✓	✓	RNA/IF/ELISA
3293	II	NO	Proliferative	45	28	✓	✓	IF
3399	II	NO	Proliferative	25	NK	✓	✓	ELISA/FLOW
3445	II	NO	Proliferative	38	28	✓	✓	RNA
3495	II	NO	Proliferative	37	32-38		✓	FLOW
3476	II	NO	Early Secretory		24-31		✓	ELISA
3478	II	NO	Early Secretory		24-31		✓	ELISA
3493	II	NO	Early Secretory	30	32-38		✓	FLOW
3172	II	NO	Mid Secretory	33	NK	✓	✓	ELISA
3482	II	NO	Mid Secretory		32-38		✓	ELISA/FLOW
3435	II	NO	NK	40	24	✓	✓	ELISA/FLOW
3340	II	NO	NK	49	NK	✓	✓	ELISA
3341	II	NO	NK	33	28	✓	✓	ELISA
3279	II	NO	NK	40	35	✓	✓	ELISA
3222	III	NO	Proliferative	35	33	✓	✓	IF/ELISA
3286	III	NO	Proliferative	38	NK	✓	✓	RNA/ELISA
3412	III	NO	Proliferative	25	30	✓	✓	RNA/ELISA/FLOW
3483	III	NO	Proliferative		24-31		✓	ELISA/FLOW

3182	III	NO	Mid Secretory	40	NK	✓	✓	ELISA
3337	III	NO	Mid Secretory	31	27	✓	✓	IF/ELISA
3358	III	NO	NK	29	30	✓	✓	ELISA/FLOW
3360	III	NO	NK	41	30	✓	✓	ELISA/FLOW
3179	III	NO	NK	29	NK	✓	✓	ELISA
3366	IV	NO	Proliferative	32	28	✓	✓	ELISA
3471	IV	NO	Proliferative		<24		✓	ELISA
3235	IV	NO	Early Secretory	28	28	✓	✓	ELISA
3349	IV	NO	Early Secretory	42	NK	✓	✓	FLOW
3489	IV	NO	Early Secretory	34	32-38		✓	FLOW
3309	IV	NO	Mid Secretory	40	30	✓	✓	ELISA
3342	IV	NO	Inactive	32	18	✓	✓	ELISA
3382	IV	NO	NK	22	30	✓	✓	ELISA/FLOW
3322	IV	NO	Menstrual	28	28	✗	✓	ELISA
3353	0	YES	Proliferative	33	28	✗	✓	FLOW
3460	0	YES	Progesterone effect	20	NK	✗	✓	FLOW
3437	0	YES	Progesterone effect	28	NK	✗	✓	FLOW
3414	0	YES	Progesterone effect	25	NK	✗	✓	FLOW
3422	0	YES	Progesterone effect	25	NK	✗	✓	FLOW
3394	0	YES	Progesterone effect	24	28	✗	✓	FLOW
3390	0	YES	Proliferative	18	30	✗	✓	FLOW
3391	0	YES	Proliferative	26	NK	✗	✓	FLOW
3418	0	YES	NK	20	NK	✗	✓	FLOW
3488	0	YES	NK	25	24-31		✓	FLOW
3371	0	YES	Early Secretory	33	32	✗	✓	FLOW
3431	I	YES	Proliferative	24	21	✓	✓	FLOW
3462	I	YES	Progesterone effect	39	28	✓	✓	FLOW
3413	I	YES	Progesterone effect	30	35	✓	✓	FLOW
3377	I	YES	Inactive	39	56	✓	✓	FLOW
3389	I	YES	NK	31	NK	✓	✓	FLOW
3473	I	YES	NK		24-31		✓	ELISA/FLOW
3465	I	YES	NK	35	NK		✓	FLOW
3403	I	YES	Mid Secretory	24	23	✓	✓	FLOW
3498	I	YES	Mid Secretory	39	<24	✗	✗	RNA/IF/ELISA/ FLOW
3361	III	YES	Progesterone effect	26	NK	✓	✓	FLOW
3428	III	YES	Mid Secretory	26	23	✓	✓	FLOW

Table 4-1 Human samples used for profiling mast cells in women with chronic pelvic pain and or without endometriosis. (NK: not known)

Unique No	AFS Grade	Exogenous hormones	H&E Stage	Age	Average Menstrual cycle length	Endometriosis	Pain	Sample Application
3491	0	NO	Proliferative	49	24-31	✗	✗	RNA/IF/ELISA / FLOW
3494	I	NO	Proliferative	31	24-31	✓	✗	RNA/IF/ELISA / FLOW
3497	0	NO	Early Secretory	34	<24	✗	✗	RNA/IF/ELISA / FLOW
3498	I	YES	Mid Secretory	39	<24	✓	✗	RNA/IF/ELISA / FLOW

Table 4-2 No pain control group samples used during Chapter 4 experiments.

4.3.2 Tissue processing

Tissue samples were either placed into 4% neutral buffered formalin (NBF) for future tissue processing and paraffin embedding (Section 2.5.1) or into RNAlater for subsequent RNA extraction (Section 2.2.1).

4.3.3 Processing of peritoneal fluids

Peritoneal fluids were centrifuged for 10 minutes at 900g at 4°C, following the World Endometriosis Research Foundation EPHect guidelines (Becker et al., 2014, Vitonis et al., 2014, Greaves et al., 2014a). The peritoneal fluid was then aliquoted and stored at -80°C until required. Cells recovered from peritoneal fluid (PF cells) were incubated with red blood cell (RBC) lysis buffer (BioLegend, USA). Briefly, cell pellets were incubated in the dark with 5ml of 1x RBC buffer for 15 minutes then centrifuged for 5 minutes at 350g at room temperature (RT). The supernatant containing the lysed red blood cells was discarded. If the resultant cell pellet was contaminated by red blood cells after lysis, the aforementioned protocol was repeated. The resultant white blood cell pellet was resuspended in 1ml of freezing medium (RPMI, Gibco, UK, with 10% dimethyl sulfoxide, DMSO, Sigma Aldrich, UK). Before storage at -80°C, cell counting and viability assessment were performed: 10µl of cell suspension was mixed with 10µl of Trypan Blue (ThermoFisher Scientific, UK), and pipetted into a disposable chamber with Countess™ II automated cell counter (AMQAX1000, ThermoFisher Scientific, UK).

4.3.4 RNA extraction from endometrial and peritoneal biopsies

RNA was extracted as detailed in Section 2.2.

4.3.5 Preparation of cDNA

Reverse transcription of RNA was performed as described in Section 2.3.

4.3.6 Quantitative Real Time PCR (Taqman® method)

The Taqman® method of polymerase chain reaction (PCR) was used for this study, gene targets, primer and probe combinations are detailed in Section 2.4.

4.3.6.1 Relative mRNA expression and statistical analysis

PCR analyses were conducted by using the standard curve method (Section 2.4.2.2) and compared to tonsil control mRNA (ASD-0088, Applied StemCell, USA), which was used as known to contain mast cells.

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, USA). Data are presented as median. One-way ANOVA was used, and Kruskal-Wallis was performed as a secondary test with Dunn's multiple comparisons test. Criterions for significance was $p<0.05$.

4.3.7 Immunofluorescence

Immunofluorescence was carried out on endometrial and peritoneal tissue sections in accordance with the standard laboratory protocols, full details in Section 2.5.10. Detection of colocalised target antigens was accomplished using the tyramide signal amplification (TSA™, details in Section 2.5.9.2, fluorophores). Primary antibody employed in chapter 4 are listed in **Errore. L'origine riferimento non è stata trovata.**.

Primary Antibody	Species raised	Source Primary Antibody	Clone	Dilution	Secondary Antibody
Tryptase	Rabbit	Abcam (ab134932)	EPR8476	1:300	1:200 - Goat anti-rabbit peroxidase (ab7171)
Chymase	Mouse	AbSerotec (MCA1930T)	CC1	1:5000	1:200 - Goat anti-mouse peroxidase (ab6823)
PAR-2	Mouse	Abcam (ab184673)	SAM11	1:500	1:200 - Goat anti-mouse peroxidase (ab6823)

Table 4-3 Primary, secondary antibodies and dilutions used in immunofluorescence.
Stock concentrations of the antibodies are reported in Table 2.5

4.3.8 Flow cytometry and FACS

4.3.8.1 Sample preparation

Following the isolation of cellular populations from peritoneal fluids (PF) (Section 4.3.3), samples were stored at -80°C. Samples were thawed at 37°C and centrifuged for 5 minutes at 1000g at 4°C. Cells were re-suspended in 1ml of FACS buffer and filtered to remove any cell clumps, using filter polystyrene FACS tubes (BD Falcon, UK). Cell suspensions were counted and viability assessed using the Countess II (AMQAX1000, ThermoFisher Scientific, UK). Human samples were incubated with AB human serum for one hour on ice (1% diluted in sterile PBS, H4522, Sigma Aldrich, UK). Samples were washed with 2 ml of FACS buffer (PBS w/o Ca^{2+} and Mg^{2+}, 2% BSA) for 5 minutes at 1000g at 4°C.

4.3.8.2 Conjugated antibody staining

Samples were stained in 100µl of FACS buffer using a panel of anti-human antibodies: epitopes, concentrations and fluorochromes are illustrated in Table 2-7. Single staining beads (eBioscience, UK) and FMOs were performed for compensation settings at the same time (detailed in Section 2.7). Cell suspensions were kept in the dark, for 1 hour on ice. Following incubation, samples were washed with 3 ml of FACS buffer and centrifuged for 5 minutes at 1000g at 4°C. To assess cell viability Dapi was used (D9542, Sigma Aldrich, UK), added at 1:500 dilution to the cell suspension, 5 minutes before running the sample through the flow cytometer or sorter.

4.3.8.3 Flow cytometry data analysis

Data gathered from flow cytometry and FACS were then analysed using the software FlowJo 8.7 (Flowjo, LLC, USA). Statistical analysis was conducted using GraphPad Prism 6.0.

4.3.9 Enzyme-linked Immunosorbent Assay

4.3.9.1 Sandwich human tryptase and chymase ELISA

The concentration of tryptase, chymase and histamine molecules in human peritoneal fluid (PF; details of PF processing in Section 2.1.2) was determined by two-site sandwich ELISA as described in Section 2.6.1. Peritoneal fluids were assayed in duplicate, information on standard samples in **Errore. L'origine riferimento non è tata trovata.** and **Errore. L'origine riferimento non è stata trovata.**.

Protein of interest	S1 (pg/ml)	S2	S3	S4	S5	S6	S7	S8
Tryptase	4000	2000	1000	500	250	125	62.5	31.2
Chymase	2000	1000	500	250	125	62.5	31.2	-

Table 4-4 Standards for tryptase and chymase sandwich ELISA.
(Tryptase: SEB070Hu, Cloud-Clone Corp., USA; Chymase: MBS2021042, MyBioSource.com USA).

Protein of interest	S1 (pg/ml)	S2	S3	S4	S5	S6	S7	S8	S9
Histamine (Kit 1)	25000	12500	62500	32000	15600	7800	3900	1900	980
Histamine (Kit 2)	100000	50000	25000	12500	6250	3200	1560	-	-

Table 4-5 Standards used for Histamine competitive ELISA kits.
(Kit 1: ENZ-KIT 140, Enzo Life Sciences, Switzerland; Kit 2: CEA927Ge, CloudClone Corp., USA).

4.3.9.2 ELISA data analysis

Data analysis was conducted using the standard curve method. The data were statistically evaluated with GraphPad Prism 6.0.

4.4 Results

4.4.1 mRNA expression of mast cell specific receptors and proteases in endometrium of women with chronic pelvic pain and endometriosis

Human mast cells are traditionally classified by the serine protease content of their granules, with tryptase and chymase being used for identification of their phenotype.

To assess expression of mast cell specific mRNAs in the eutopic endometrial tissue of women with chronic pelvic pain with/without endometriosis homogenates were analysed using RT-PCR. Concentrations of *TPSAB1* (gene encoding human tryptase α and β isoform) mRNAs were significantly lower in grade 0 patient group (chronic pain, no endometriosis) when compared to the control group. Endometrial biopsies from women with endometriosis (grade I-III) appear to have a lower expression of *TPSAB1* mRNA compared to the control group, but higher compared to the grade 0 group but this did not reach significance (Figure 4-2-A). A similar expression pattern was seen for *CMA1* (gene encoding human chymase), mRNAs which were significantly downregulated in grade 0 eutopic endometrium and lower in grade I-III when compared to no pain control group (Figure 4-2-B).

The study was extended to investigate the expression of genes encoding the protease-activated receptor 2 (*PAR-2*) and the histamine receptor 1 (*HR1*) on biopsies (Figure 4-2-C/D). Interestingly, mRNA expression of these receptors that may be activated by tryptase or histamine derived from mast cells are also down-regulated in grade 0 and grade I-III samples.

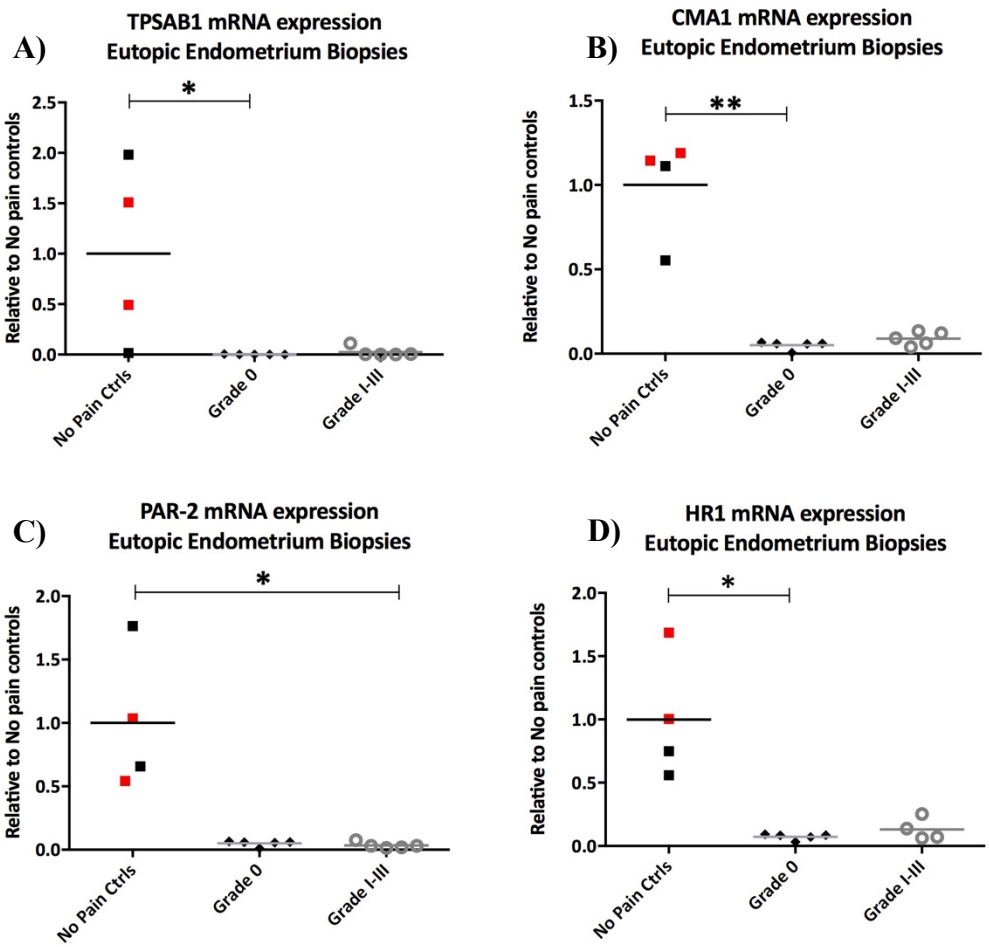

Figure 4-2 Concentrations of mast cell proteases and effector receptors mRNAs were altered in eutopic endometrium of women with chronic pelvic pain and/or endometriosis.
A-B-D) *TPSAB1* (tryptase), *CMA1* (chymase) and *HR1* (histamine receptor): mRNA expression levels were significantly downregulated in eutopic endometrium from women with pelvic pain, and lower levels were detected in endometrial biopsies from women with endometriosis. **C)** The mRNA expression of *PAR-2* (protease-activated receptor 2) was instead significantly downregulated in eutopic endometrium from women with different grades of endometriosis. Data shown as median, statistical test used was One-way ANOVA, Kruskal-Wallis post-test, *p<0.05, **p<0.01. No pain controls n=4, red dots indicate control patients with asymptomatic endometriosis, grade 0 n=5, grade I-III n=5.

Expression of mast cell mediators and receptors was also examined in tissue homogenates from endometriotic lesions (Figure 4-3). *TPSAB1* and *HR1* mRNA concentrations in lesions was lower when compared to no pain control eutopic endometrium (Figure 4-3-A,D). *PAR-2* mRNA expression was significantly downregulated in endometriotic lesions (Figure 4-3-C). A different expression pattern

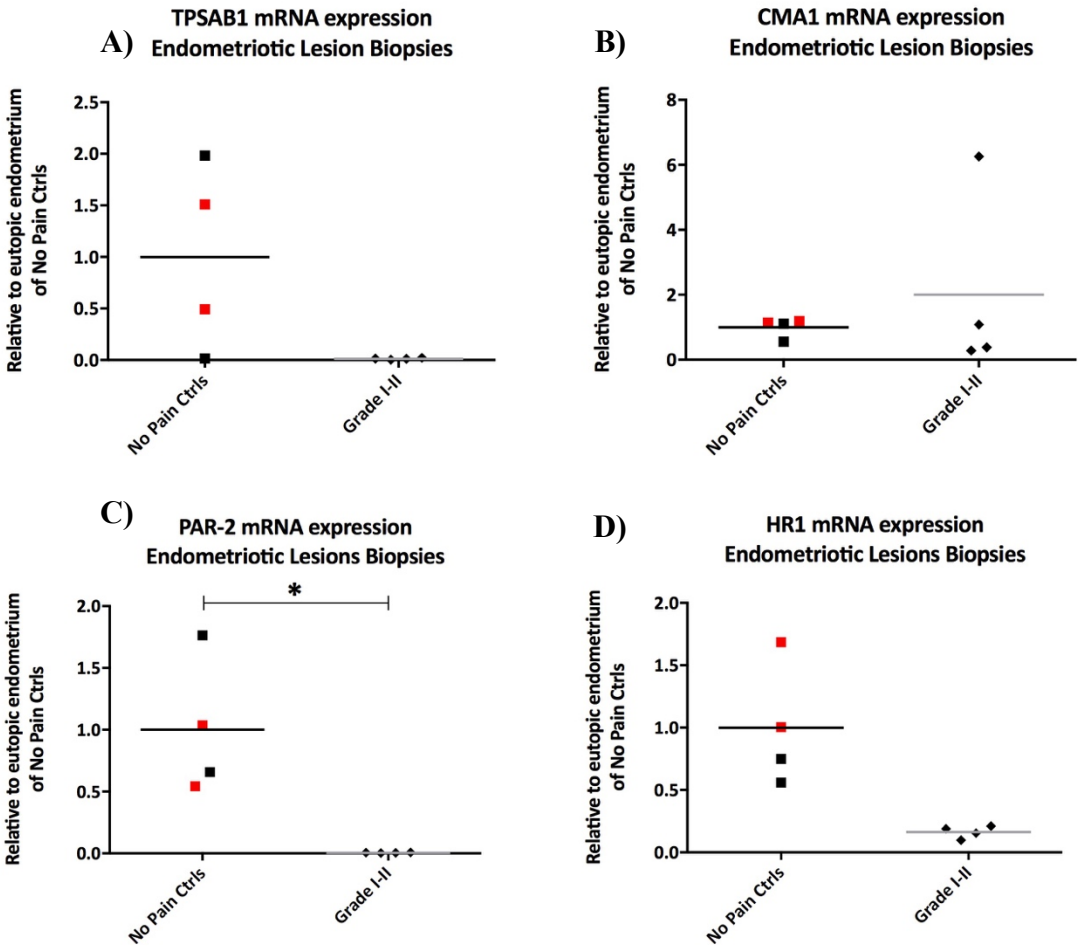

Figure 4-3 Expression of receptors and proteases of mast cell mRNAs in endometriotic biopsies. **A-B-D)** mRNA concentrations of mast cell proteases were not significantly different in endometriotic lesions compared to eutopic endometrium from control women (no pain group). *TPSAB1* and *HR1* expression resulted lower and *CMA1* mRNA was instead higher in lesion homogenates. **C)** *PAR-2* receptor expression was significantly downregulated in endometriotic lesions. Data shown as median, statistical test used was One-way ANOVA, Kruskal-Wallis post-test *p<0.05, No pain controls n=4, red dots indicate control patients with asymptomatic endometriosis, endometriotic lesions n=4.

was found when assessing the mRNA concentrations of *CMA1* mRNAs with a non-significant trend towards increase in lesions compared to controls (Figure 4-3-B).

The investigation of expression of mRNAs was expanded by exploring concentration of mast cell related genes in peritoneal tissue homogenates. Two kinds of peritoneum biopsies were taken during diagnostic laparoscopy: one from the "prone" site of the peritoneum where lesions are usually formed in a region named the pouch of Douglas (recto-uterine pocket) and one from the front pelvis peritoneum, the opposite site to where endometriotic lesions are usually found. These two samples were designated as peritoneum "prone" and peritoneum "distant" to the usual sites of endometriotic lesions. Tissue biopsies from the same locations were collected from the "no pain" control group patients.

TPSAB1 mRNA concentrations were significantly lower in peritoneal "distant" tissue homogenates from women experiencing chronic pelvic pain (grade 0) and from those also affected by endometriosis (grade I-III, Figure 4-4-A) compared to the no pain control group peritoneum. *CMA1* expression did not change across the different groups (Figure 4-4-B). Grade I-III had one sample outlier, with very high *CMA1* mRNA concentrations. The expression of receptors for mast cell mediators remained unchanged between the control group and chronic pain groups. Message RNAs of *PAR-2* and *HR1* appeared unchanged in grade 0 and I-III when compared to the no pain controls (Figure 4-4-C/D).

Similarly, in peritoneal prone biopsies, the expression profile for mast cell mediators and receptors showed an apparent downregulation of *TPSAB1* mRNA concentrations in grade 0 which was significant the grade I-III group, compared to no pain controls (Figure 4-5-A). *CMA*, *PAR-2* and *HR1* mRNAs did not vary within the different patient groups (Figure 4-5-B,D).

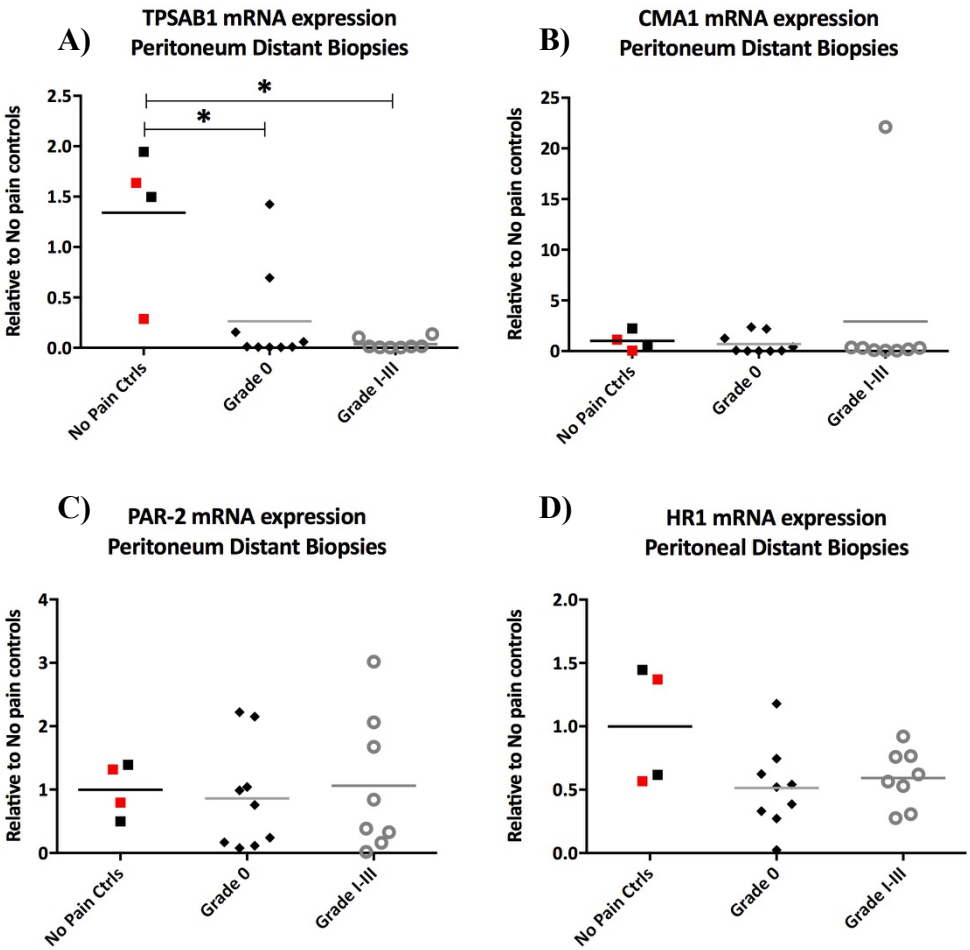

Figure 4-4 Investigation of mast cells mediators and receptors in distant peritoneum in women with chronic pelvic pain and/or endometriosis.
A) mRNA expression of *TPSAB1* was significantly altered in women experiencing chronic pelvic pain group and endometriosis group when compared to No pain group in peritoneal distant biopsies, *p<0.05. **B-C-D)** Gene expression of *CMA1*, *PAR-2* and *HR1* remained unchanged within the groups. Data shown as median. No pain controls n=4, red dots indicate control patients with asymptomatic endometriosis, grade 0 n=9, grade I-III n=8.

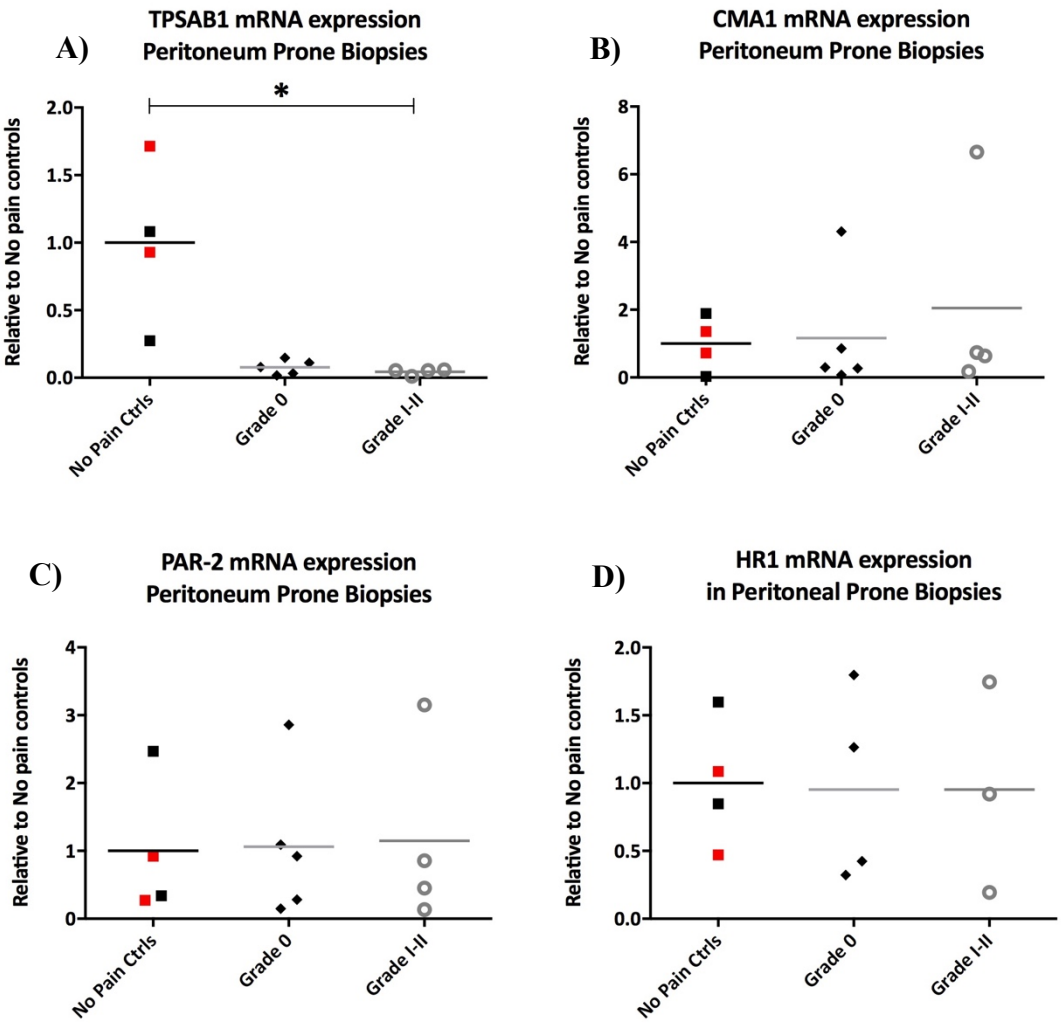

Figure 4-5 mRNA expression of mast cells related genes in peritoneal prone biopsies from women attending the pain clinic.

A) *TPSAB1* gene was significantly downregulated in peritoneum prone from women with endometriosis, **B-C-D)** *CMA1*, *PAR-2* and *HR1* mRNA expression remained unchanged within the different groups of patients and controls. Data shown as median, statistical test used was One-way ANOVA, Kruskal-Wallis post-test *$p<0.05$, No pain controls n=4, red dots indicate control patients with asymptomatic endometriosis, grade 0 n=5, grade I-II n=3-4.

4.4.2 Profiling of mast cells in women attending a pain clinic identified changes in subtypes compared to controls

Expression of mast cell proteases were also investigated at the protein level, by visualising their localization using immunofluorescent staining in all patient groups. Due to the high degree of patient variability, results are shown patient by patient for the "no pain" group and two representative patients of proliferative and secretory phase are illustrated for the group 0 and group I-III datasets.

The following results are discussed based on the patient groups and different phases of the menstrual cycle. Starting with a sample collected during the proliferative phase from a control group patient (3491) (Figure 4-6), chymase positive mast cells were detected in the peritoneal tissue biopsies but were absent in the functional layer

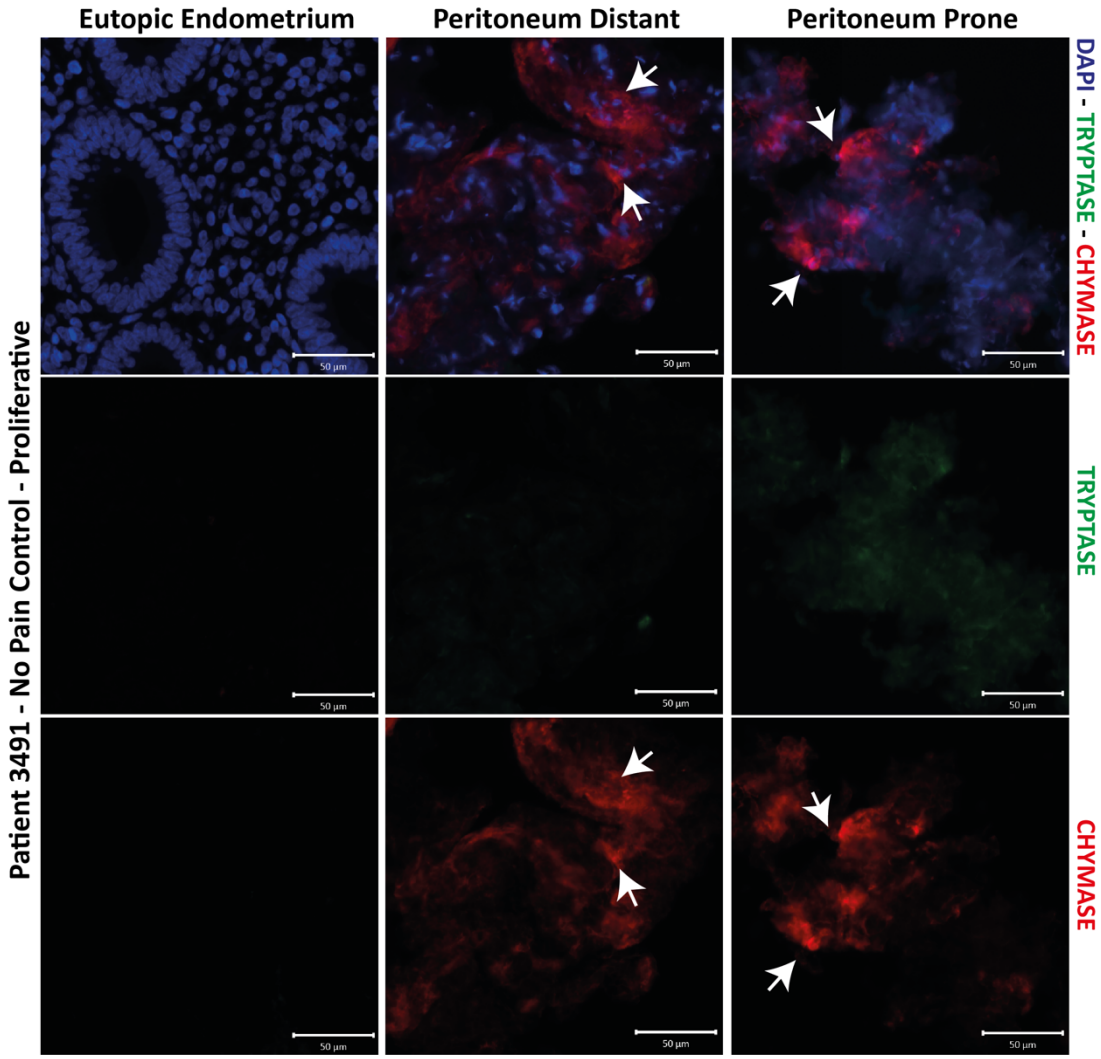

Figure 4-6 Identification of mast cell subtypes in eutopic endometrium and peritoneal biopsies of a control patient (no pain and no endometriosis).
Mast cells were not detectable in the functional endometrial biopsy, MCs resulted chymase only phenotype and highly activated in both peritoneal biopsies. (White arrows: MC_C cells)

of the endometrium. Interestingly, in this patient the peritoneal mast cells expressed chymase but not tryptase, suggesting there were of the MC_C subtype. Furthermore, they appeared to be highly activated, as there was abundant extracellular immunostaining of chymase. This finding was consistent in both distant and prone peritonea.

In contrast, mast cells were detected in the functional endometrium of patient 3494, who was also in the proliferative phase (Figure 4-7), and in this sample two different subtypes were reported: MCs positive for tryptase and chymase (MC_{TC}) and the very rare endometrial type, chymase only (MC_C). Mast cells present in the endometrium appeared to be in a "resting" state where serine proteases are retained inside the cytoplasm. Remarkably, mast cells of the MC_{TC} subtype had apparently degranulated and chymase was widely spread in the peritoneal biopsies. Patient 3494 suffered from grade I asymptomatic endometriosis.

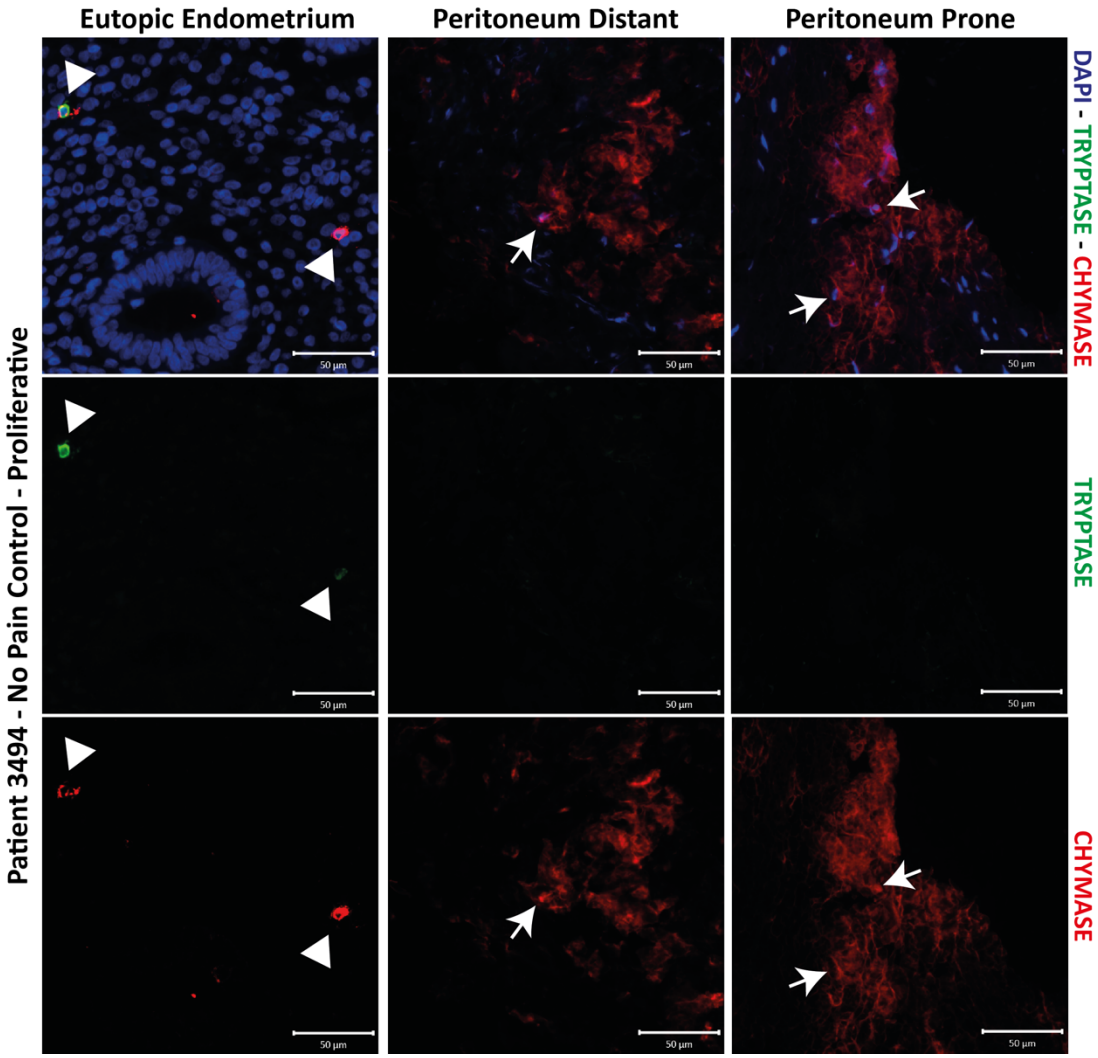

Figure 4-7 Mast cell identification in endometrial and peritoneal biopsies of a proliferative sample from no pain group.
Mast cells resulted immunopositive for tryptase and chymase, and chymase positive in the eutopic endometrium, and were found in the resting phase (no extracellular release of proteases). MCs were instead highly activated and singly positive for chymase in both peritoneal biopsies, distant and prone to lesion. Need to be specified, patient 3494 had asymptomatic grade I endometriosis, therefore it was included in the "no pain" control group. White triangle indicates tryptase/chymase double positive MC. (White triangles: MC_{TC}, white arrows: MC_{C})

The MC phenotype was detected different during the secretory phase in the functional endometrium; cells were single positive for tryptase and in a resting state (Figure 4-8, Figure 4-9), a finding that was in line with the results reported in Chapter 3. Mast cells in the peritoneal wall were again activated and intensively immunopositive for chymase (Figure 4-8). Patient 3498, presented with grade I asymptomatic endometriosis and showed mast cells degranulating chymase in the peritoneum prone however MCs were detected with lower intensity in the distant peritoneal biopsies (Figure 4-9).

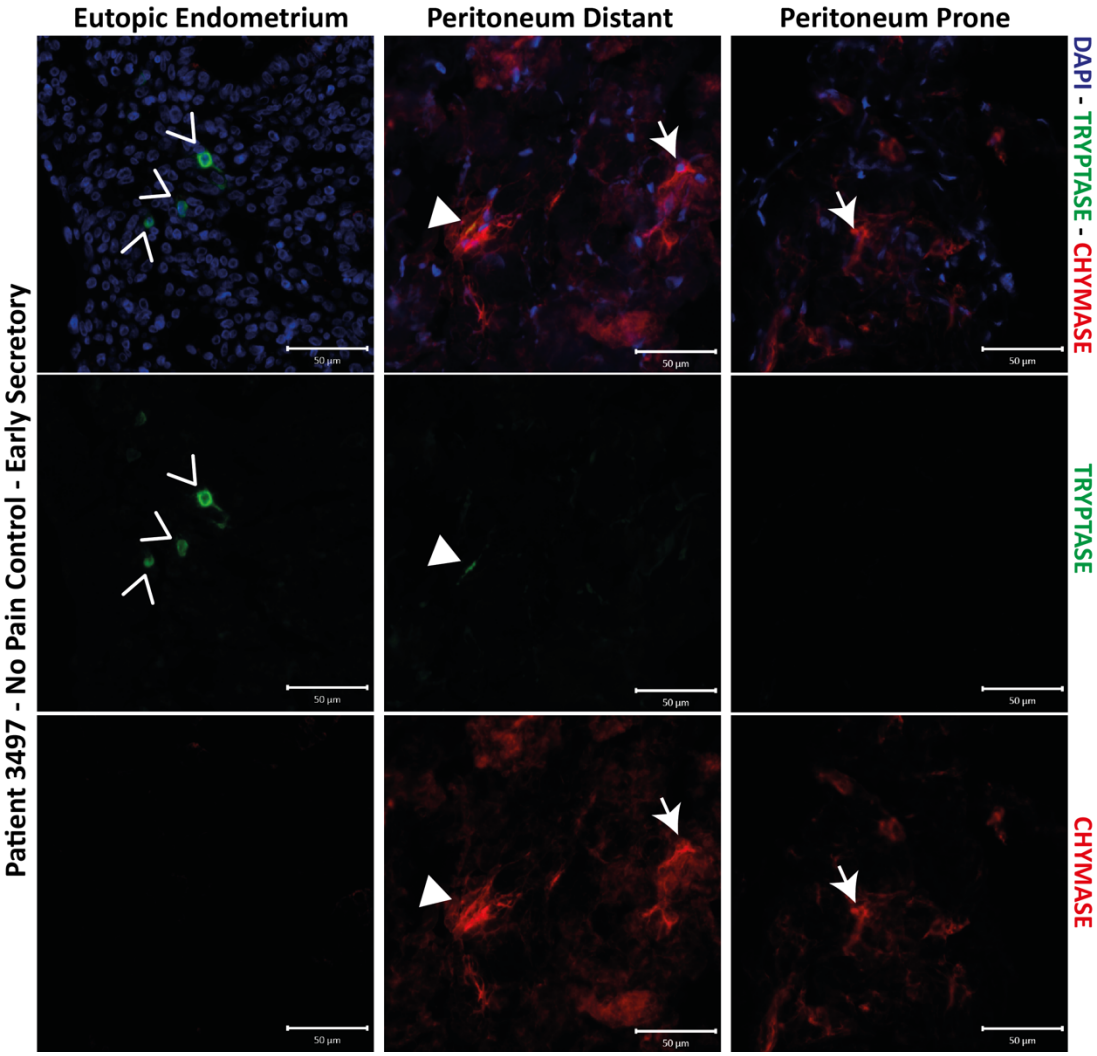

Figure 4-8 Mast cells identification in a "no pain" control patient during secretory phase: MC subtype found in the eutopic endometrium corresponded to the typical functional endometrial MC type, tryptase single positive, intensively investigated in Chapter 3. Peritoneal MCs were detected degranulating chymase in both "distant" and "prone" wall (White triangles: MC_{TC}, white Vs: MC_T; white arrows: MC_C).

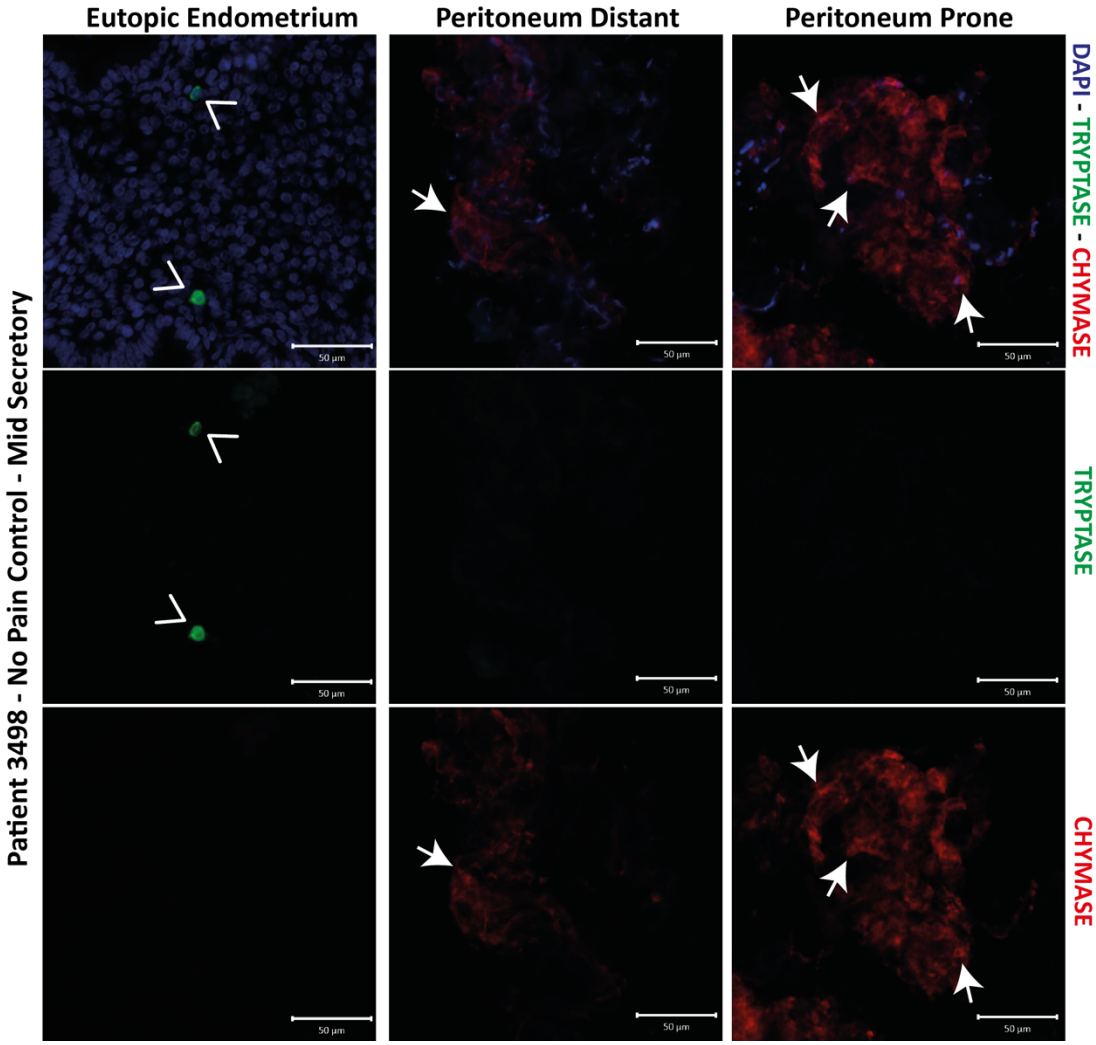

Figure 4-9 Immunolocalization of mast cells in a secretory phase patient with asymptomatic endometriosis.
Endometrial MCs showed the "classical" functional endometrial phenotype: tryptase single positive, as described in Chapter 3. MCs were not detectable in the peritoneal distant, but were instead activated and chymase positive in the peritoneum prone biopsy. (White triangles: MC_{TC}, white Vs: MC_T; white arrows: MC_C).

MCs were also investigated in the group of patients affected by chronic pelvic pain who had no evidence of endometriosis at the time of laparoscopy, (grade 0, Figure 4-10/14). In a proliferative sample 3298 (Figure 4-10), MCs were tryptase and chymase positive in the functional endometrium, showing an activated state, with chymase staining in the extracellular matrix. Compared to "no pain" controls, MCs were difficult to detect in the peritoneal samples, and the chymase and tryptase staining were reduced and in some cases absent, for example in the peritoneum prone of this patient (Figure 4-10).

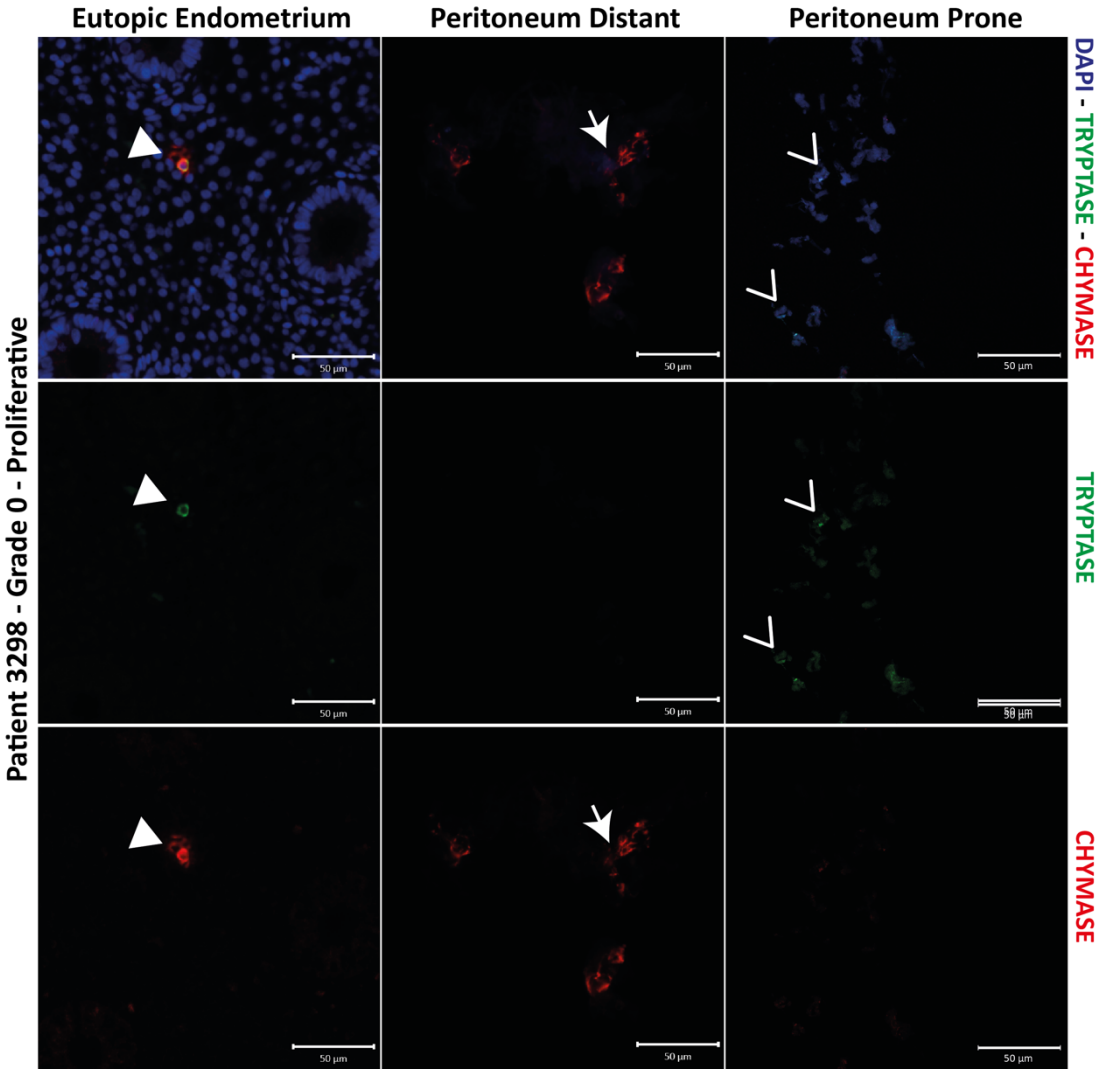

Figure 4-10 Mast cell localization in one patient during proliferative phase affected by chronic pelvic pain and no endometriosis.
Endometrial MCs resulted double positive for tryptase and chymase, with little extracellular chymase staining. Peritoneal MCs, when detectable, were single chymase or tryptase positive, with a lower staining if compared to "no pain" controls. Grade 0 patients during proliferative phase n=3. (White triangles: MC_{TC}, white Vs: MC_T; white arrows: MC_C)

A similar result was shown by immunofluorescence in biopsies from patient 3282; MCs were detectable with low expression of chymase, retained in the cytoplasm. Moreover, peritoneal MCs were detected that were double positive for both tryptase and chymase in the peritoneum prone (Figure 4-11). MCs were not detectable in the proliferative functional endometrium.

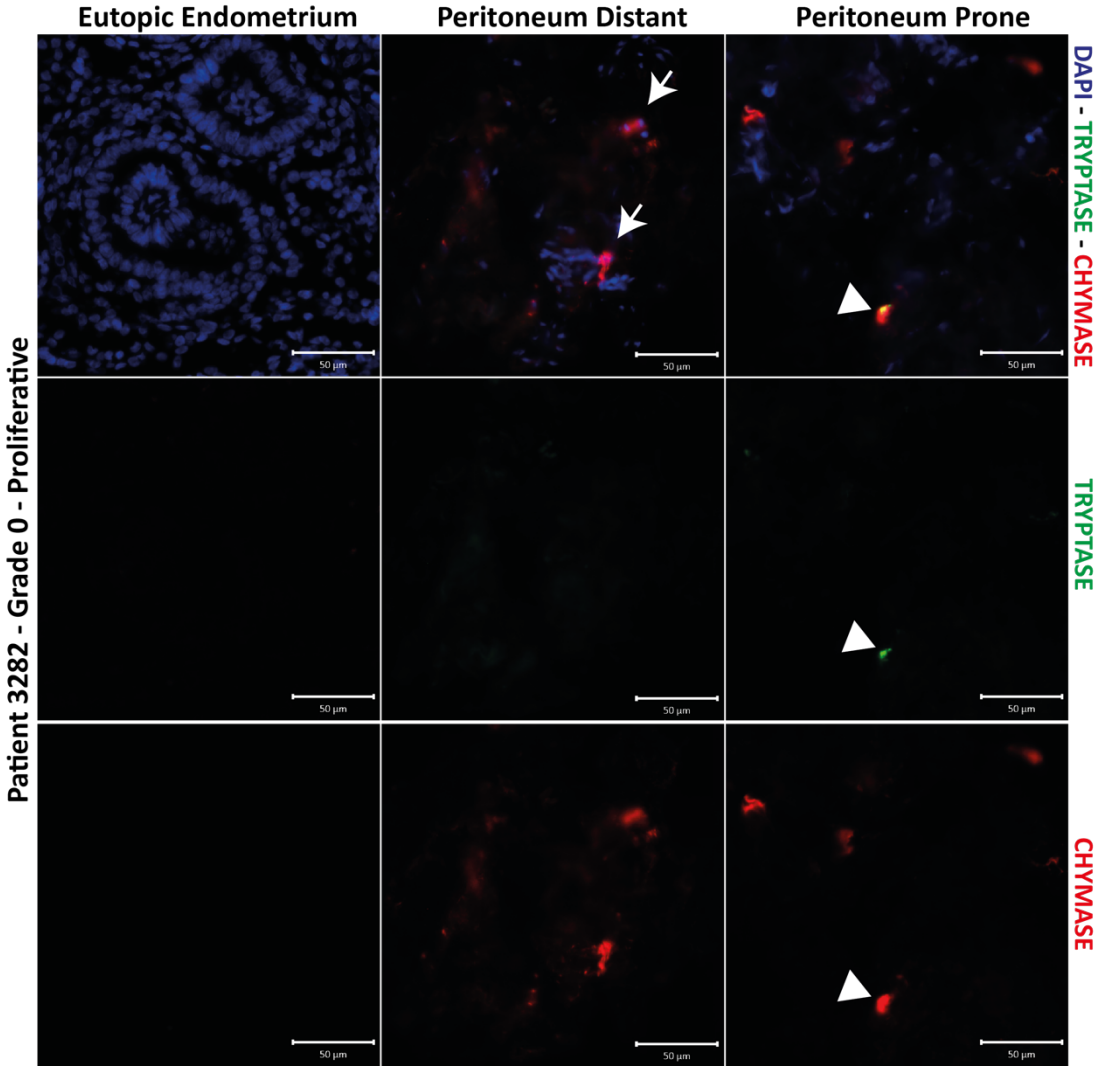

Figure 4-11 Tryptase and chymase staining in tissue biopsies from a chronic pelvic pain patient during proliferative phase.
MCs were not detectable in the eutopic endometrial biopsy. Peritoneal MCs were MC_C in the distant peritoneum and MC_{TC} in peritoneum prone. Grade 0 patients during proliferative phase n=3. (White triangles: MC_{TC}, white Vs: MC_T; white arrows: MC_C).

Two representative samples were selected to describe the MC phenotype in chronic pelvic pain patients during the secretory phase; the total number of patients used in this study was four. During the secretory phase, interrogation of biopsies from women experiencing chronic pelvic pain (no endometriosis) revealed they had all three MC subtypes present: tryptase only (MC_T), tryptase and chymase (MC_{TC}) (Figure 4-12, Figure 4-13) and chymase only (MC_C) (Figure 4-13) in the functional layer of their endometrium. MCs were chymase positive (MC_C) and in a degranulating state in the peritoneum distant.

Chymase staining was less intense when compared to the "no pain" control images, described above. Interestingly, chymase staining was not only detected in mast cells, but also in cuboidal cells, at the edge of the tissue suggesting these might be mesothelial cells (Figure 4-13). In peritoneal prone biopsies (Figure 4-12, Figure 4-13), chymase staining was more specific to single MCs. Moreover, tryptase staining was detected in peritoneal MCs, in line with the finding in proliferative samples consistent with the MC_{TC} phenotype.

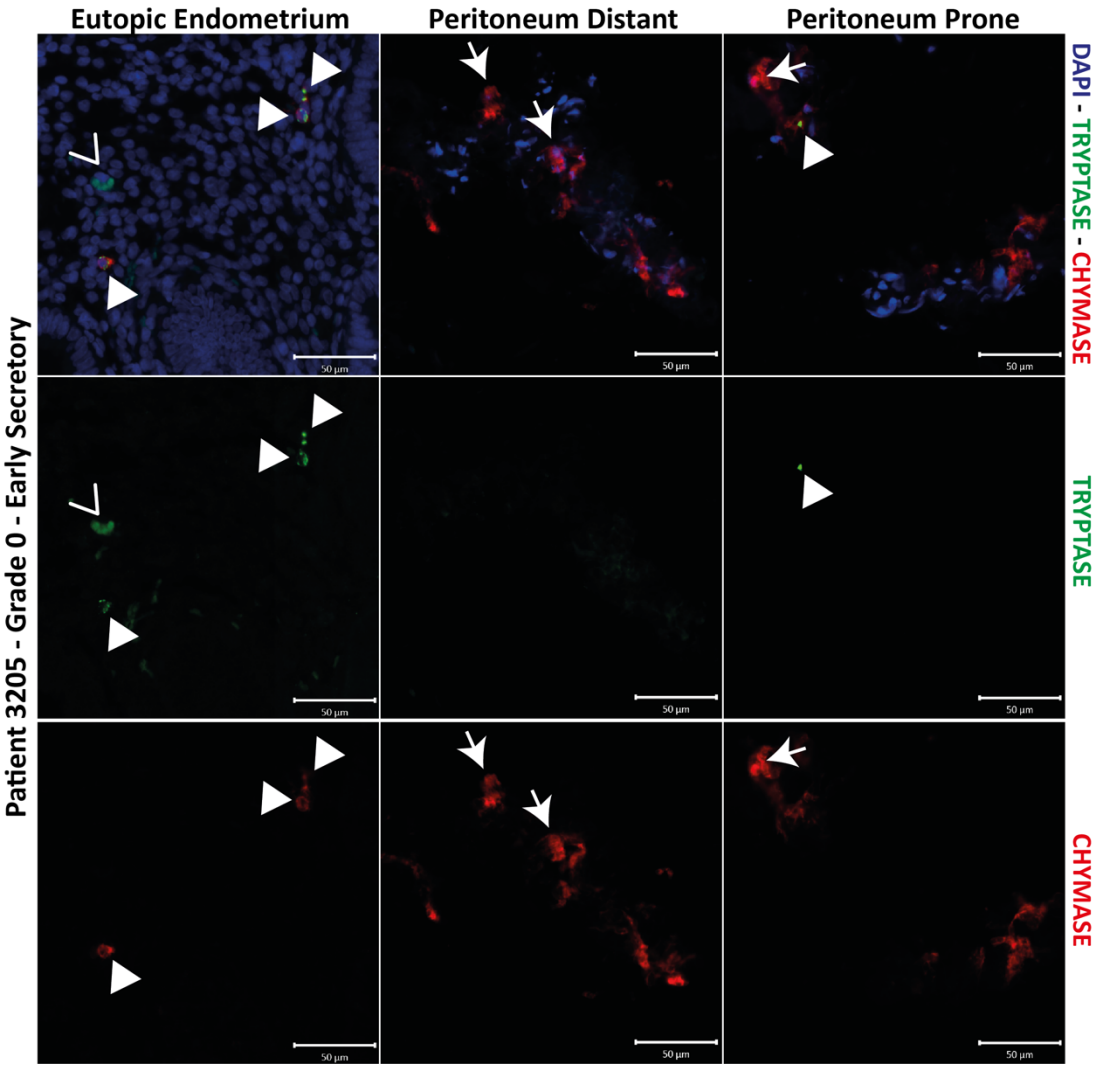

Figure 4-12 Mast cells detection in endometrial and peritoneal biopsies from a representative secretory phase sample from chronic pelvie pain group..
Two subtypes of MCs were found in the eutopic endometrium: MC_T and MC_{TC}. MCs resulted degranulating and MC_C type in both the peritoneal biopsies. Grade 0 patients during secretory phase n=4. (White triangles: MC_{TC}, white Vs: MC_T; white arrows: MC_C).

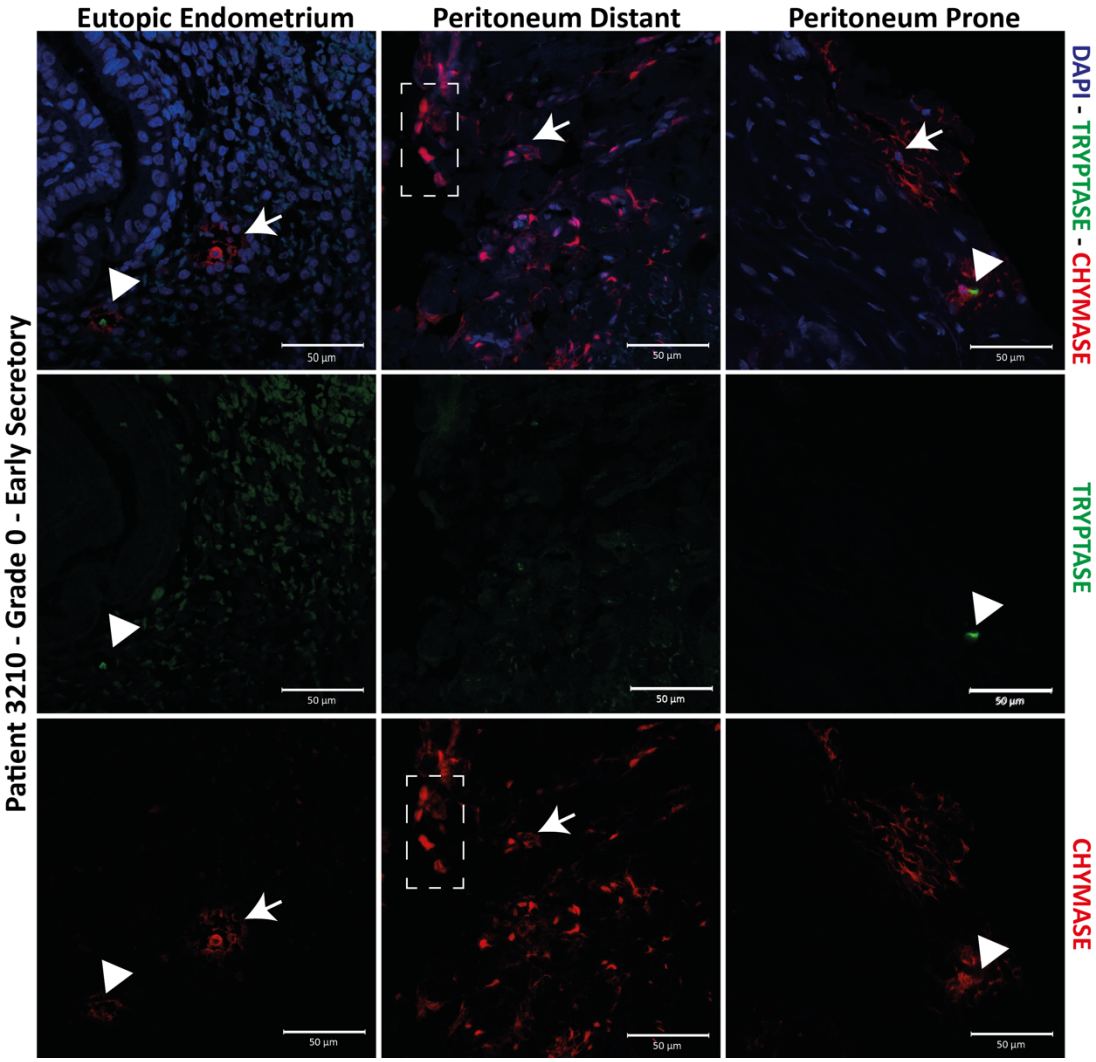

Figure 4-13 Identification of mast cell in eutopic endometrium and peritoneal wall in a representative secretory phase sample from chronic pelvic pain group.
Endometrial MCs were found MC_C and in the activated state, with extracellular staining. Different types of cells resulted positive for chymase in the peritoneum distant, cuboidal cells together with mast cells, suggesting mesothelial cells (white rectangle). Grade 0 patients during secretory phase n=4. (White triangles: MC_{TC}; white arrows: MC_C).

To complete the dataset, double immunofluorescence for mast cell proteases, tryptase and chymase, was carried out on samples from women classified as suffering from different grades of endometriosis (grades I-III). To display the results for each group two representative samples were selected to cover both the proliferative and secretory phase of the menstrual cycle within this group. Two MC subtypes were detected in the functional layer of the endometrium during the proliferative phase; MC_{TC} and MC_C (Figure 4-14, Figure 4-15). The staining pattern suggested that endometrial mast cells were in the "resting" state, with proteases contained in cytoplasmic granules. Peritoneal MCs showed phenotypical heterogeneity in the selected samples; MCs were predominantly chymase positive with a small portion of tryptase/chymase positive in prone peritoneum. The intensity of the immunostaining varied from "distant" to "prone" peritoneal wall being higher to lower respectively. MCs were also successfully detected in the ectopic endometrial-like lesions, with

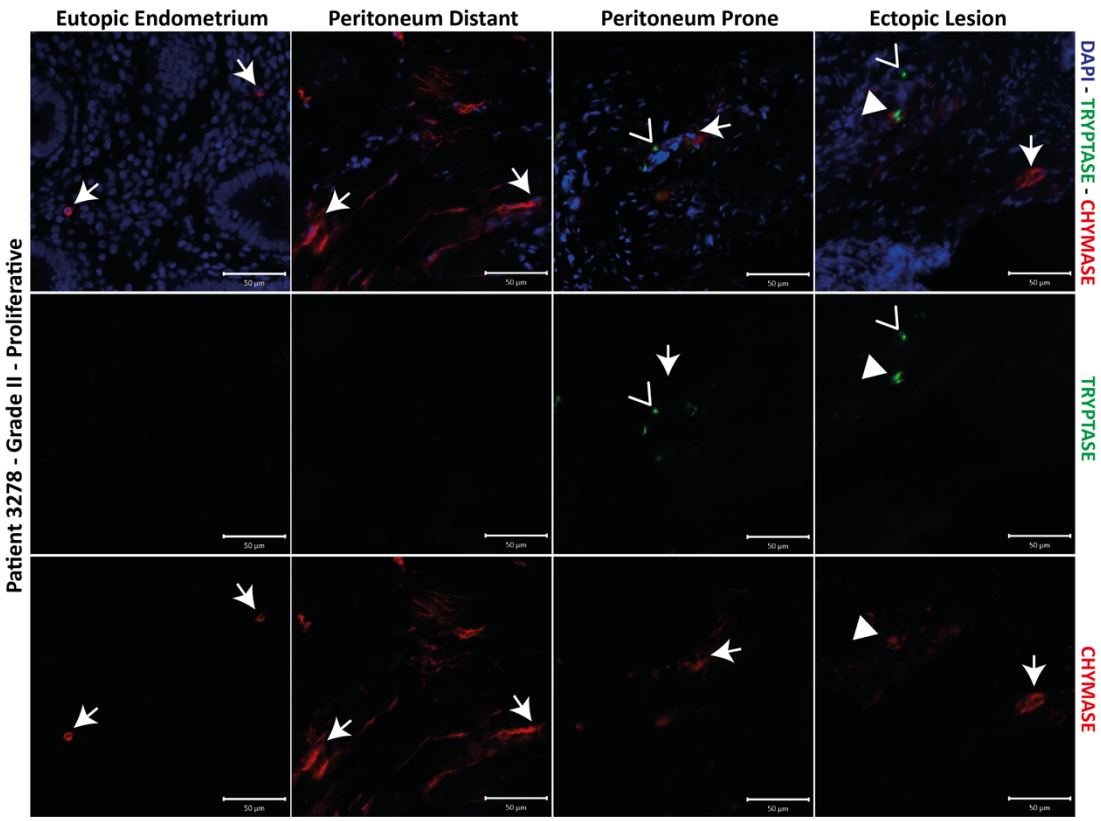

Figure 4-14 Detection of mast cell mediators in grade II endometriosis tissue biopsies.
Chymase was the most predominant serine protease detected in the different tissue samples from a patient with grade II endometriosis during the proliferative phase. Tryptase was detected with lower intensity in MCs in peritoneum prone and ectopic lesion. Overall, the expression of MC mediators was lower if compared to "no pain" control. Grade I-III patients during proliferative phase n=4. (White triangles: MC_{TC}, white Vs: MC_T; white arrows: MC_C).

intense positive staining for chymase. The MC state detected in the different peritoneal biopsies was considered as "activated".

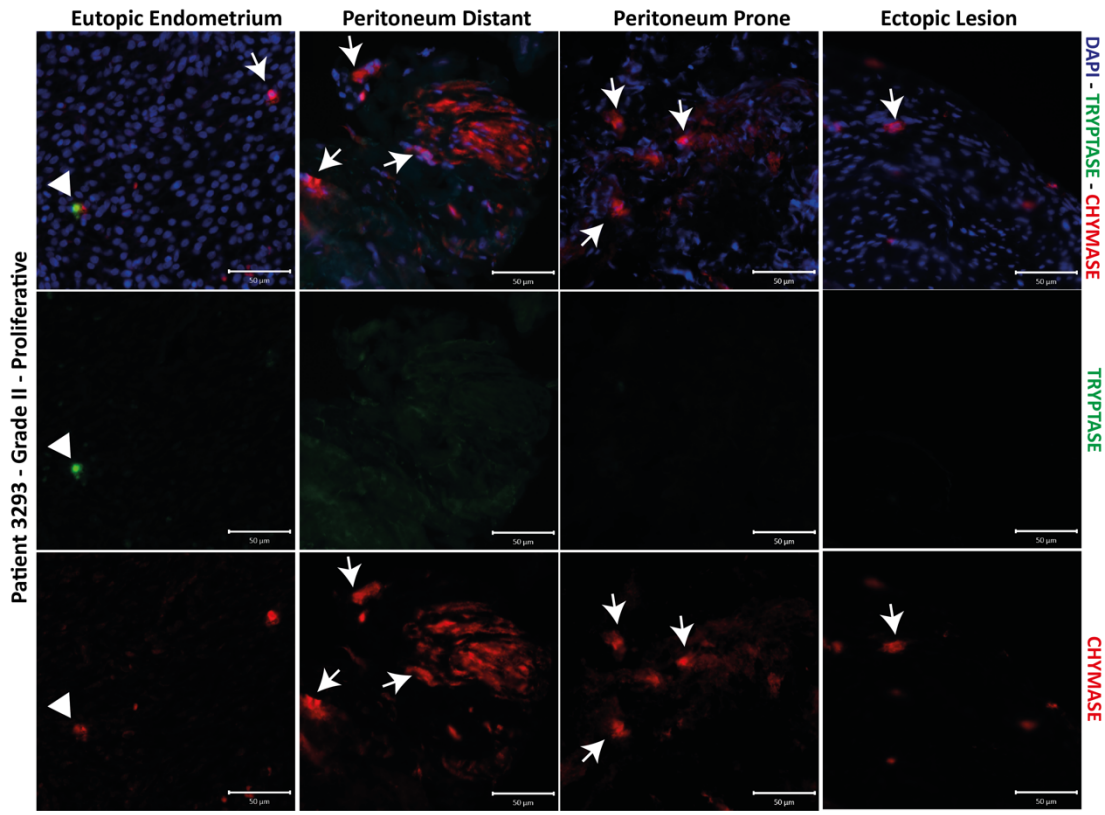

Figure 4-15 A representative sample for MC identification in a proliferative grade II patient. In the eutopic endometrium MCs were MC_{TC} and MC_C. MC_C phenotype was retained also in the ectopic lesions. MCs in peritoneal biopsies showed highly activated chymase single positive phenotype. Grade I-III patients during proliferative phase n=4. (White triangles: MC_{TC}, white arrows: MC_C)

Interestingly, MCs were not detectable in the eutopic endometrium of any of the secretory samples from women with endometriosis screened during this study (Figure 4-16, Figure 4-17). Furthermore, the MC phenotype changed in the peritoneal samples recovered during the secretory phase, both single positive MC_C, as well as MC_{TC} were also detected. Notably, both the intensity and quantity of the staining appeared reduced in comparison to the "no pain" control peritoneal samples. Chymase detection was restricted specifically to the mast cell population, with a lower activation/degranulation profile. MCs were found in a relatively high number in peritoneum prone and lesion biopsies.

Findings of this sections are summarised in Table 4-6.

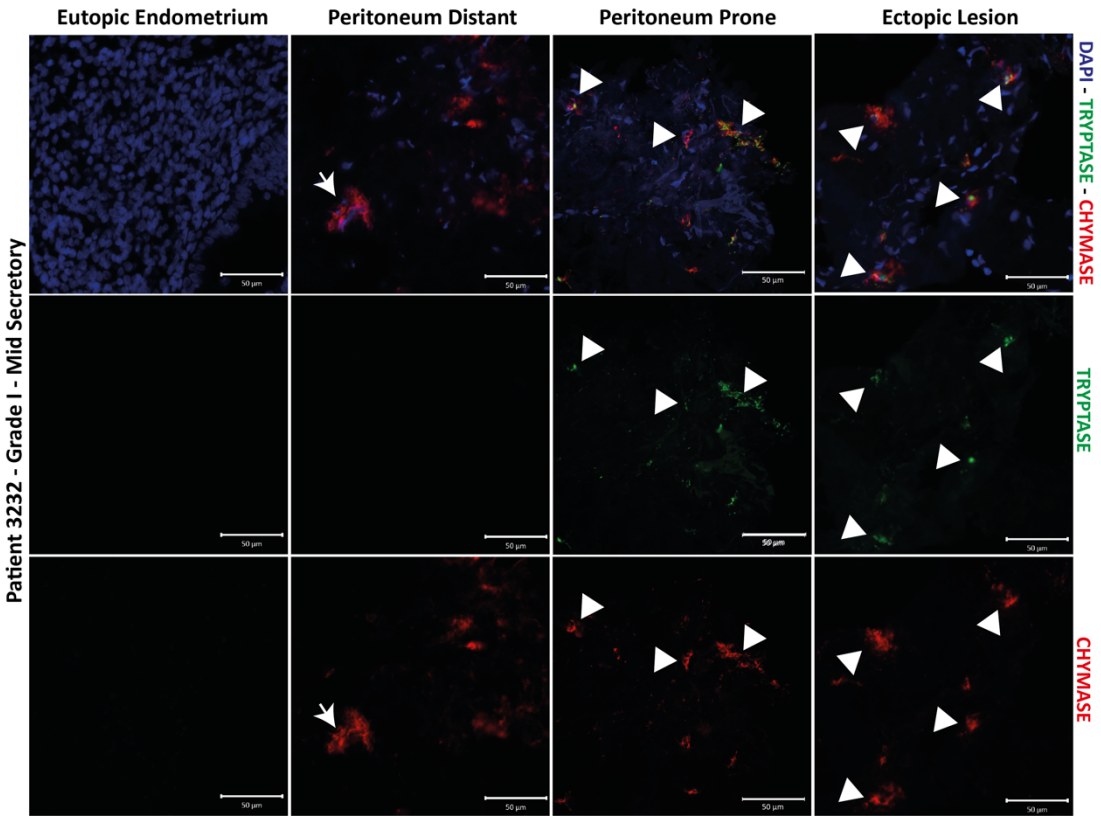

Figure 4-16 Tryptase and chymase staining on a representative secretory sample set of endometriosis group.

MCs were detected in the peritoneal biopsies and endometriotic lesions. Chymase was the most prevalent protease in the MC granules. Tryptase was detected in a large number of peritoneal MCs which was different to results in the other patient groups. Grade I-III patients during secretory phase n=4. (White triangles: MC_{TC}, white arrows: MC_{C}).

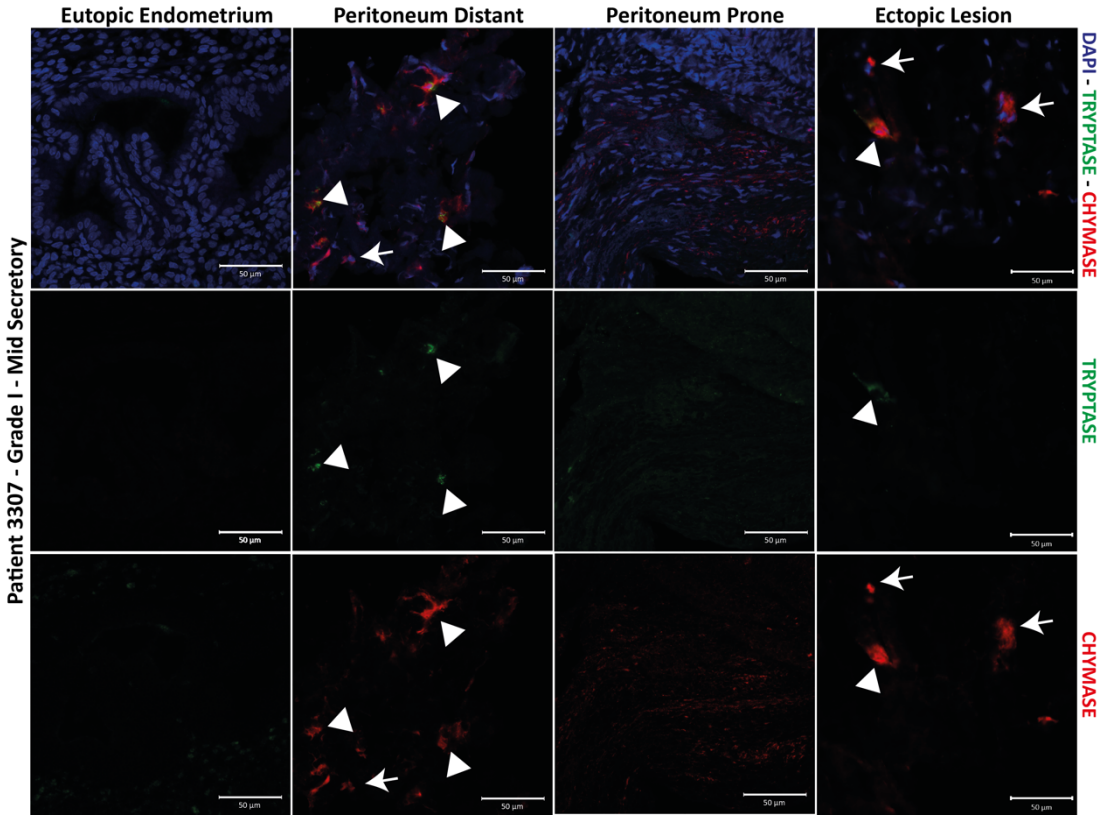

Figure 4-17 Double positive MCs in representative samples from patient with endometriosis sample in secretory phase.
MCs were present in peritoneum distant and ectopic lesion biopsies: MC_{TC} and MC_{C}. MCs resulted activated with extracellular chymase; tryptase was retained in the cytoplasm. Grade I-III patients during secretory phase n=4. (White triangles: MC_{TC}; white arrows: MC_{C}).

	Proliferative phase				Secretory phase			
	EE	**PD**	**PP**	**L**	**EE**	**PD**	**PP**	**L**
CTRLs	MC_{C} MC_{TC}	MC_{C} MC_{TC}	MC_{C} MC_{C}	—	MC_{T}	MC_{C}	MC_{C}	—
Grade 0	MC_{TC}	MC_{C}	MC_{T} MC_{TC}	—	MC_{T} MC_{TC}	MC_{C}	MC_{TC}	—
Grade I-II	MC_{C} MC_{TC}	MC_{C}	MC_{C} MC_{TC}	MC_{C} MC_{TC}	nd	MC_{C} MC_{TC}	MC_{TC}	MM_{C} MC_{TC}

Table 4-6 Summary table of MC phenotype in tissue samples studied in this project.
EE: eutopic endometrium, PD: peritoneum distant, PP: peritoneum prone, L: lesion, nd: not detected

4.4.3 Identification of protease-activated receptor 2 (PAR-2) in the peritoneum of women suffering of chronic pelvic pain and endometriosis.

PAR-2 receptor can be activated by MC proteases and in this study it was for the first time, it was immunolocalized in the human peritoneal lining, both in women with and without chronic pelvic pain. PAR-2 protein expression was dependent on the phase of the menstrual cycle, and more intense in secretory phase compared to proliferative phase samples, as shown in Figures 17/18/19. Notably, this was independent of the site of the biopsies, in prone or distant peritoneum. This result is in line with the endometrial expression of *PAR-2* described in Chapter 3, Section 3.4.2, which demonstrated that *PAR-2* mRNA and protein expression was unregulated during the secretory phase. Figure 4-18 illustrates the immunolocalization of PAR-2 protein in the peritoneum of "no pain" control women. Specifically, PAR-2 was detected in some but, not all the dapi positive cells; no tryptase positive MCs were detected consistent with the results detailed in Section 4.4.2.

Remarkably, PAR-2 immunoexpression appeared strongly upregulated in the peritoneum from women with chronic pelvic pain, both in proliferative and secretory phases of the menstrual cycle, as shown in Figure 4-19. One exception to this pattern was noted in peritoneum "distant" biopsies, as PAR-2 protein expression appeared similar in intensity to that of "no pain" controls, both during proliferative and secretory phases (see Figure 4-18). Interestingly, tissue sections of peritoneum distant from patient 3210 showed that putative mesothelial cells also express PAR-2 protein (cuboidal cells indicated by white arrows in Figure 4-19).

Furthermore, a striking pattern of PAR-2 protein expression was detected in the peritoneal biopsies from women with pelvic pain and endometriosis, including those containing samples with endometriotic lesions (Figure 4-20). There was no difference in the expression between the phases of the cycle, the location or the type of biopsies. PAR-2 was expressed homogenously in all the tissue sections, suggesting a ubiquitous upregulation throughout the peritoneal cavity of women suffering of endometriosis and pelvic pain. In addition, tryptase positive cells were detected in the different samples, as reported in Section 4.4.2.

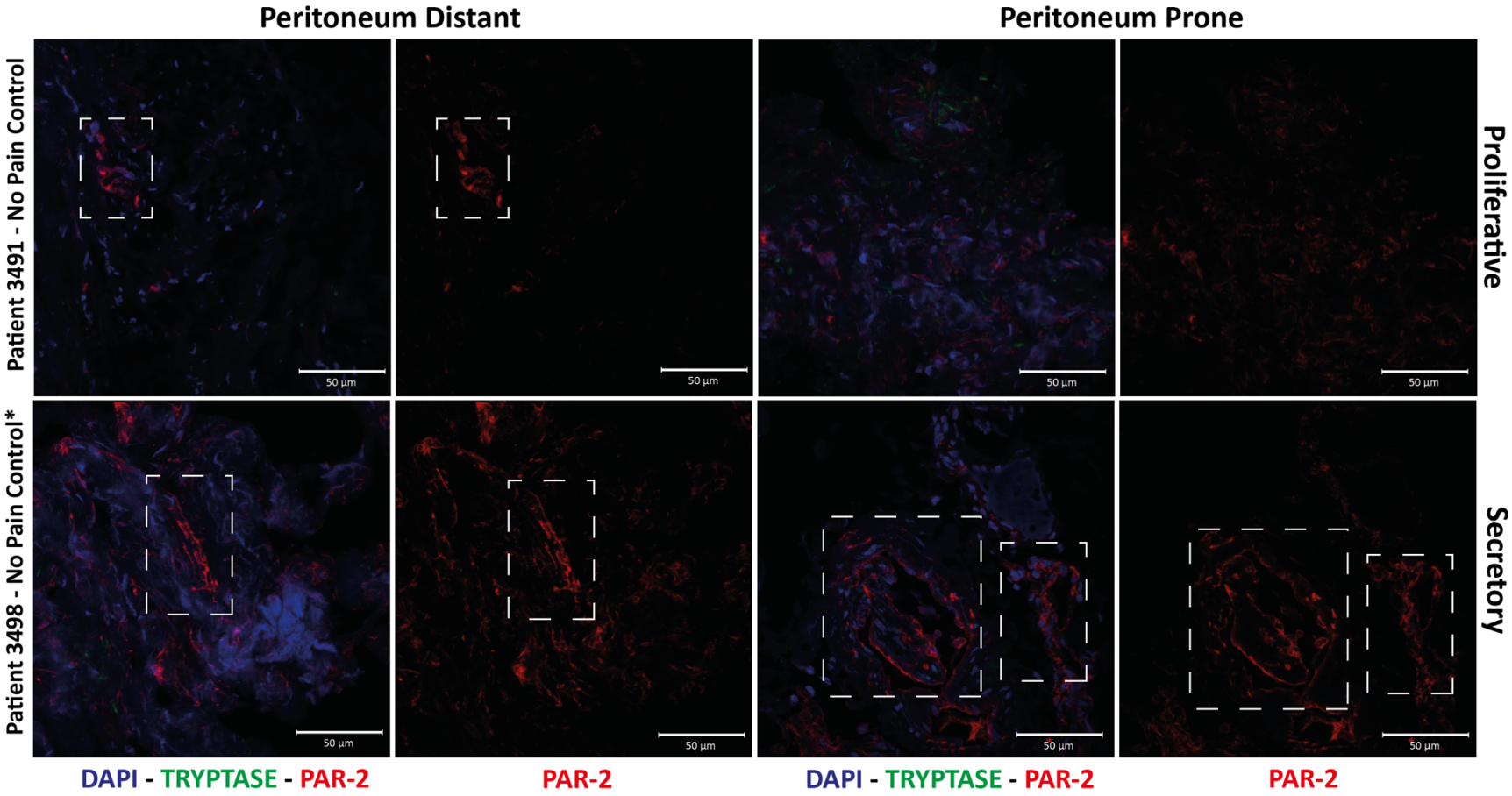

Figure 4-18 Protease-activated receptor 2 (PAR-2) immunoexpression during proliferative and secretory phase in the peritoneal lining of "no pain" control women.

Double immunofluorescence showed that PAR-2 expression was higher during secretory phase and confirmed that the absence of tryptase positive mast cells in the peritoneal environment. White rectangles: cuboidal mesothelial cells .Proliferative phase n=2, secretory phase n=2.

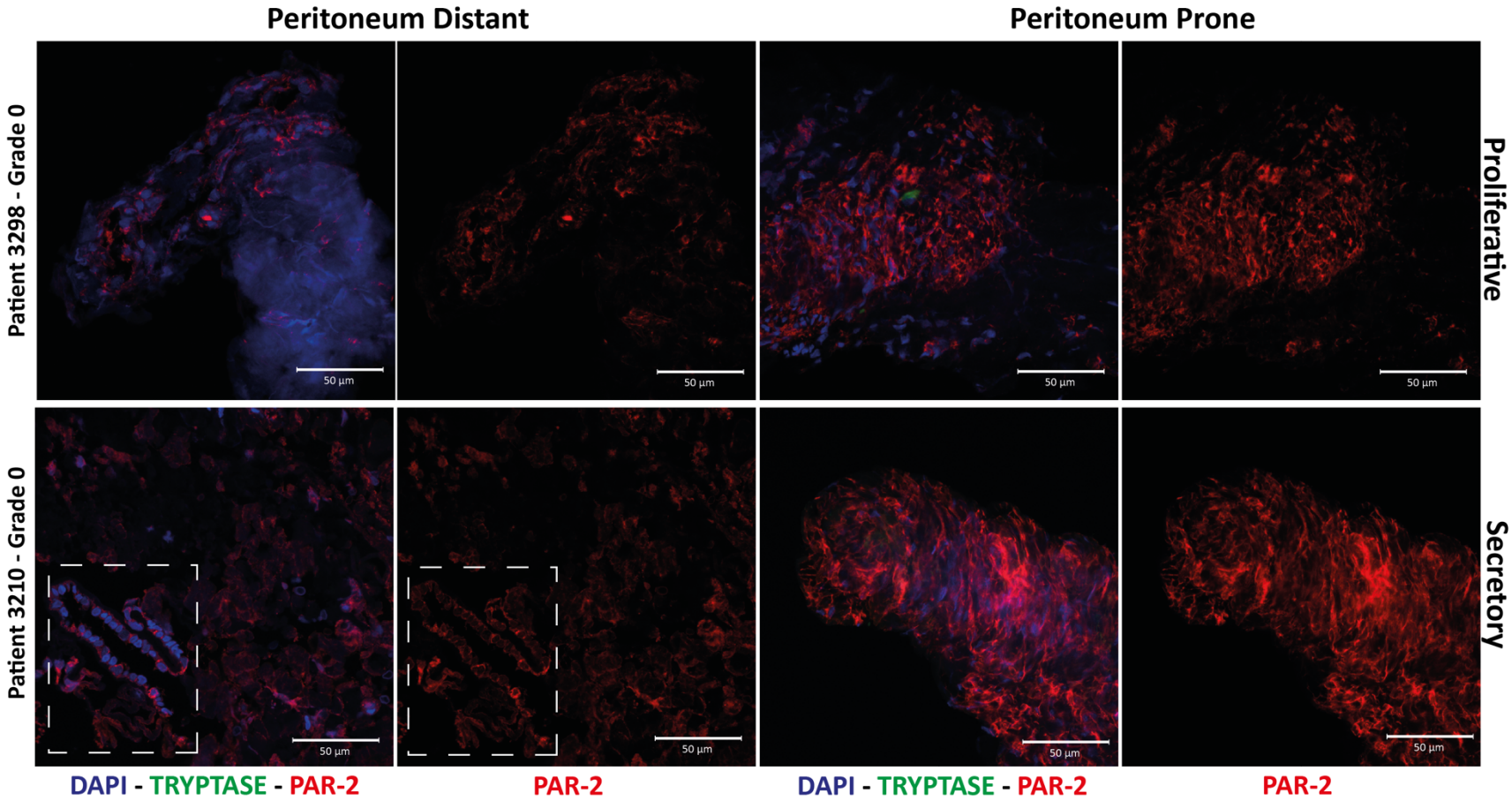

Figure 4-19 PAR-2 expression in peritoneal biopsies from women suffering from chronic pelvic pain.
PAR-2 expression appeared higher in peritoneal prone biopsies compared the distant peritoneum. Expression did not change during different phases of the menstrual cycle. White rectangles: cuboidal cells, possible mesothelial nature. Proliferative n=2, secretory n=2.

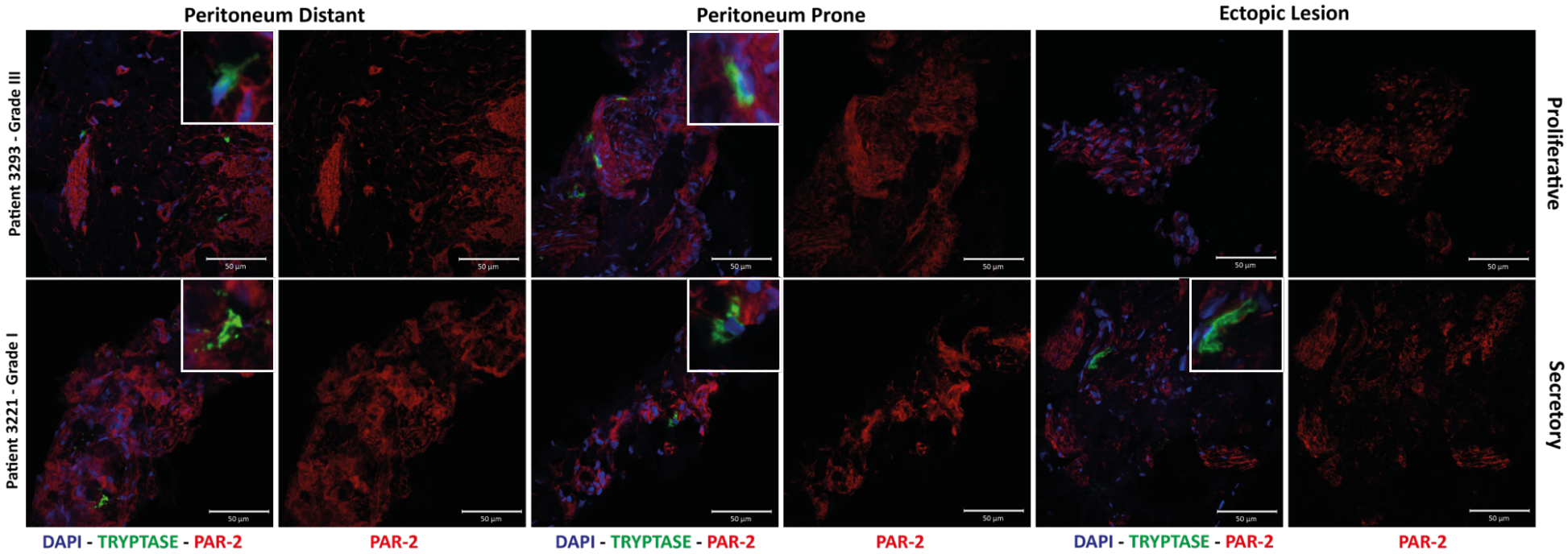

Figure 4-20 Representative images of PAR-2 expression in the peritoneal cavity from women affected by endometriosis and chronic pelvic pain.
PAR-2 protein was detected ubiquitously in the peritoneal cavity of women with endometriosis and chronic pain, in both peritoneal biopsies and endometriotic lesions. PAR-2 immunoexpression appeared higher compared to the control group (Figure 4-18). Tryptase positive mast cells were also localized within the different tissue sections. Proliferative n=2, secretory n=2.

	Proliferative			Secretory		
	PD	**PP**	**L**	**PD**	**PP**	**L**
CTRLs	↑	↑	__	__	↑	__
Grade 0	↑	↑↑↑	__	↑↑	↑↑↑	__
Grade I-II	↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑	↑↑

Table 4-7 Summary table for intensity of PAR-2 staining with immunofluorescence.
↑: low intensity, ↑↑: medium intensity and ↑↑↑: high intensity of fluorescence signal.

4.4.4 Profiling of peritoneal fluid of women with and without endometriosis, identifying mast cells within the immune cell population.

4.4.4.1 Experimental protocol for isolation of peritoneal fluid MCs

Due to the limited literature on the quantification of mature mast cells by flow cytometry, the current investigation started with the optimization of a mast cell specific antibody panel for flow cytometry. Mast cells differentiate from the common leukocyte antigen positive cells ($CD45^+$) as well as other immune cells, such as macrophages, granulocytes and lymphocytes. To accomplish an accurate quantification of mast cells within all the other $CD45^+$ derived immune cells, it was necessary to establish exclusion criteria for the gating strategy, as shown in Figure 4-21. Mast cells were quantified by c-kit/FcεRIα^{++} (Figure 4-21-G), when monocytes ($CD14^+$), T and B lymphocytes ($CD3^+$, $CD19^+$), natural killer ($CD56^+$), granulocytes ($CD11b^+$) and macrophages (CD11b-$HLADR^{++}$, $CD163^+$) were excluded.

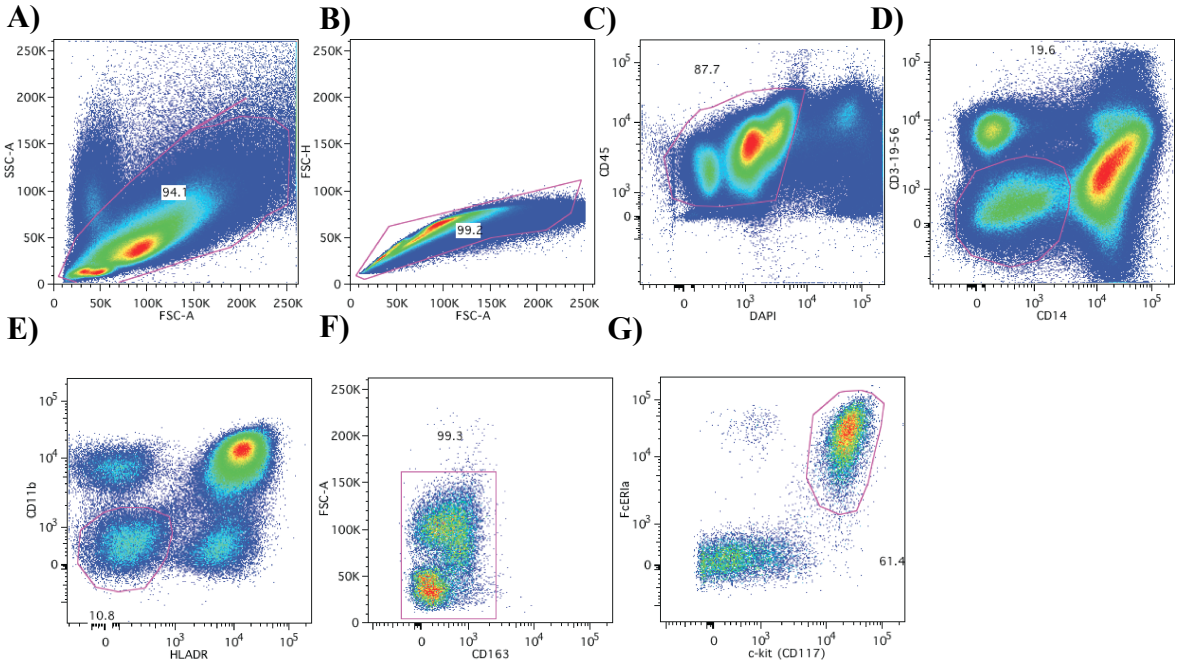

Figure 4-21 Representative gating strategy used during flow cytometry and sorting experiments on human peritoneal fluid isolated white cells.
Mast cells belong to CD45$^+$ population as well as B, T and NK cells, monocytes and macrophages. Thereby the gating strategy used for quantifying mast cells in the peritoneal fluid cell population during the current study, followed exclusion criteria. After size and doublets exclusion **(A-B)**, cells were gated on CD45$^+$ and dapi negative **(C)**, CD3-19-56 (T-B-NK cells) and CD14 (monocytes) negative **(D)**. For excluding macrophages, cells were gated on CD11b-HLADR negative **(E)** and CD163 negative **(F)**. Finally, mast cells were identified using the unique combination of c-kit (CD117) and FcεRIα antibodies, showed in panel **G**. These gates were then applied to all the subsequently analysed samples.

4.4.4.2 Analysis of MC populations in peritoneal fluids from women with or without endometriosis

In addition to MCs, the percentage of macrophages was also analysed as there is the extensive evidence of their role in endometriosis (Bacci et al., 2009, Capobianco and Rovere-Querini, 2013), they were considered a useful comparator.

MCs represented a small percentage of the total live CD45$^+$ cells in the peritoneal fluid (median <0.2%), whilst macrophages accounted for a greater proportion at being between 10 and 20% of live CD45$^+$ cells; there was considerable variation between individuals (Figure 4-22). Figure 4-22-A,B illustrates the frequency of MCs and macrophages in the peritoneal fluid from women not on hormonal treatments. The median percentage (middle line of box and whisker plot) of both macrophages and MCs did not change between control and pain/endometriosis groups.

Interestingly, the distribution of values in the third quartile (values above the median) was higher in the minimal/mild endometriosis group (grade I-II) for macrophages and in the moderate/severe endometriosis group (grade III-IV) for MCs, although in neither case they did not reach statistical significance due to the wide variation between individuals. In peritoneal fluid of women undergoing hormonal therapy, macrophage and MC distribution showed a different pattern: the percentage of macrophages appeared to have a trend of non-significant increase in patient groups of grade 0 and grade I-II. In contrast, grade III-IV values were lower when compared to no pain controls, however not reaching statistical significance (Figure 4-22-C). MC median distribution remained unchanged between the difference groups (Figure 4-22-D).

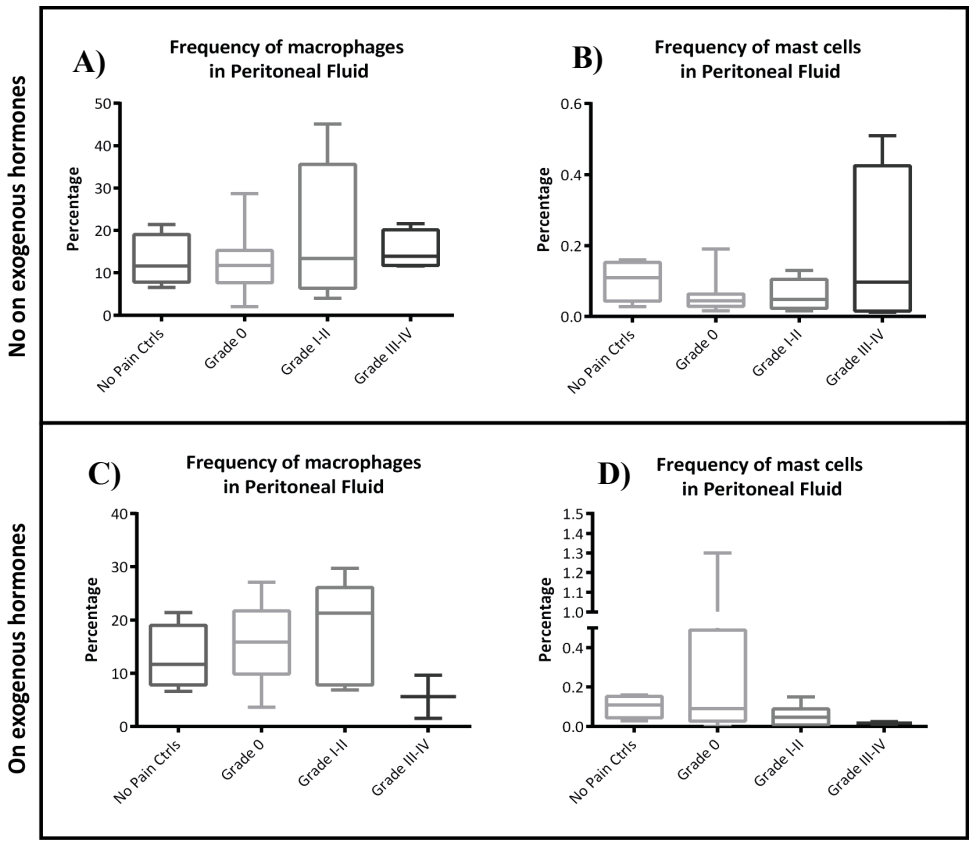

Figure 4-22 Frequency of macrophages (HLADR-CD11b $^{++}$) and mast cells (c-kit-FcεRIα $^{++}$) in live CD45^{+} cells from the peritoneal fluid isolated cells.

Macrophages and mast cells were detectable in the peritoneal fluid (PF), macrophages represented a higher cell population compared to mast cells, in average 10% and 0.1% respectively. The median of distribution of both macrophages and mast cells did not change across the different groups. Interestingly, higher percentage of macrophages was detected in patients with chronic pelvic pain and minimal/mild endometriosis **(A)**, and percentage of mast cells was higher in more severe endometriosis grades, such as III-IV **(B)**. No pain controls n=4, grade 0 n=10, grade I-II n=9, grade III-IV n=4. Panel **C** showed a different pattern of macrophage frequency in the PF from women under hormonal therapy, an increase in group 0 and group I-II, although it did not reach statistical significance.

4.4.5 ELISA analysis of mast cell proteases and histamine in peritoneal fluid from women with pelvic pain and endometriosis

Mast cell specific protease, tryptase and chymase were quantified in the peritoneal fluid from control, pelvic pain and endometriosis patient groups. Tryptase was detected at the median value of 1075.12 pg/ml in peritoneal fluid of the "no pain" control group (Figure 4-23-A); median values concentrations appeared lower in the pain group (grade 0), and in the minimal/mild (grade I-II) and moderate/severe endometriosis groups, but this was not statistically significant (Table 4-1). Concentrations of chymase remained unchanged between the different groups, with an median value of 461 pg/ml in the peritoneal fluid of control women (Table 4-1, Figure 4-23-B). Moreover, the presence of histamine was also investigated in the peritoneal fluid samples. To complement the studies on MC granule-related mediators, concentrations of histamine (MCs and basophils) were measured. Histamine concentrations varied significantly between the different groups, being significantly lower in group 0 and groups III-IV compared with no pain controls ($p<0.05$, Figure 4-23-C).

Median pg/ml	No Pain Ctrls	Grade 0	Grade I-II	Grade III-IV
Tryptase	1075.12	122.83	737.33	727.33
Chymase	461	434	454	188
Histamine	151019	41634	44323	29941

Table 4-1 Median values of protease and histamine concentrations in the peritoneal fluid.

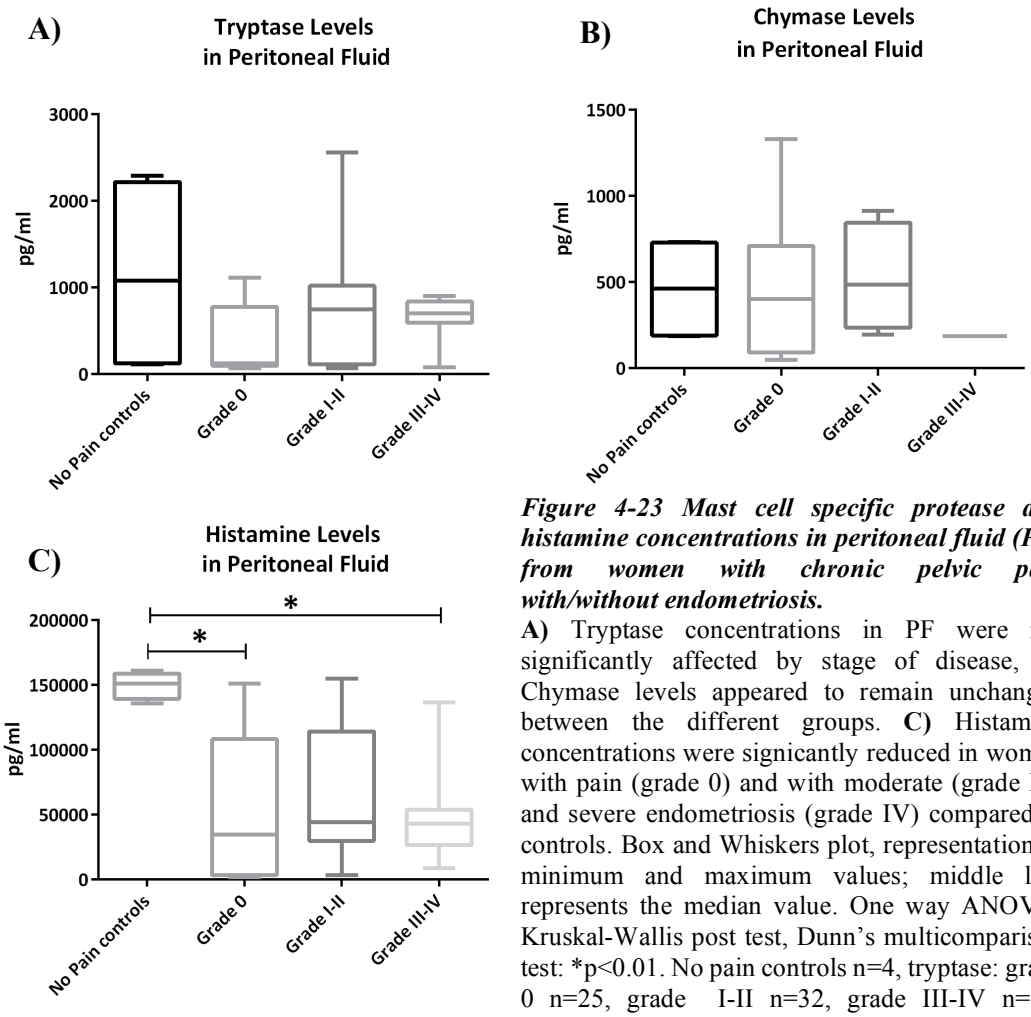

Figure 4-23 Mast cell specific protease and histamine concentrations in peritoneal fluid (PF) from women with chronic pelvic pain with/without endometriosis.

A) Tryptase concentrations in PF were not significantly affected by stage of disease, **B)** Chymase levels appeared to remain unchanged between the different groups. **C)** Histamine concentrations were signicantly reduced in women with pain (grade 0) and with moderate (grade III) and severe endometriosis (grade IV) compared to controls. Box and Whiskers plot, representation of minimum and maximum values; middle line represents the median value. One way ANOVA, Kruskal-Wallis post test, Dunn's multicomparison test: *$p<0.01$. No pain controls n=4, tryptase: grade 0 n=25, grade I-II n=32, grade III-IV n=16; chymase: grade 0 n=13, grade I-II n=15, grade III-IV n=1; histamine: grade n=21, grade I-II n=30, grade III-IV n=15.

4.5 Discussion

Endometriosis is a chronic inflammatory disease characterized by the growth of endometrial-like lesions outside of the uterus, mainly in the peritoneal cavity (Giudice and Kao, 2004). The disease affects approximately 10–15% of women of a reproductive age (Simoens et al., 2012). Impairments in the decidualisation response, a transformation event that the stromal compartment of the endometrium must undertake to host pregnancy (Gellersen and Brosens, 2014), have often been reported in the eutopic endometrium of women with endometriosis (Wei et al., 2009). The leukocyte populations within the eutopic endometrium and ectopic lesions have been extensively described (Jones et al., 1998, Bacci et al., 2009, Braundmeier et al., 2012). These studies suggested that alterations in macrophages, T-cells and uterine natural killers might contribute to the pathology of the disease and its correlated infertility and chronic pelvic pain but less attention has been paid to the other immune cell subtypes.

A few studies have reported MC infiltration into the peritoneum of women with endometriosis (Fujiwara et al., 2004, Kempuraj et al., 2004, Sugamata et al., 2005). However, MCs have not previously been investigated in the eutopic endometrium of women experiencing chronic pelvic and/or endometriosis. The current study is, to my knowledge, the first one to investigate the MC phenotype in the functional endometrium from women with pain and/or endometriosis.

As demonstrated in the previous chapter, MCs are members of the uterine leukocyte population. Although, MCs do not appear to fluctuate in numbers during the menstrual cycle, and they are detectable in all the uterine compartments, from the myometrium to the functional endometrium. The largest numbers of MCs are detected in the myometrium and the basal endometrium and MCs are quite rare in the functional endometrium. In normal uterus, MCs have a tryptase/chymase phenotype (MC_{TC}) in myometrium and basal endometrium, and tryptase only phenotype (MC_T) in the functional endometrium, a finding in agreement with Jeziorska et al. (1995) and following the standard mast cell classification system based on their granule contents (Pejler et al., 2007). Furthermore, the rare MC_C subtype represented a small number of resident uterine MCs, specifically it was detected in the basal endometrial and myometrial layers.

In the current study, immuno-profiling of the functional endometrium of women with chronic pain and of women with endometriosis detected MCs with MC_{TC} or MC_C phenotype, a result that was in contrast with the MC phenotype detected in control "no pain" control samples, and samples used during Chapter 3. This abnormal MC protease phenotype was also detected in two of the "no pain" control samples, 3494 and 3498, in patients who suffered from asymptomatic endometriosis. I speculate that this altered endometrial MC phenotype is consistent with the concept of phenotypical plasticity of MCs (Galli et al., 2011), which stated that MCs can mature and adapt based on their surrounding microenvironment. The presence of MC_{TC} and MC_C is consistent with reports that the endometrial microenvironment is altered in women with endometriosis. Further, this finding might suggest the potential of invasiveness of the shed endometrium from women with endometriosis. It has been demonstrated that both tryptase and chymase can exert angiogenic effects. For example, it has been shown that tryptase may cause significant augmentation of capillary growth (Caughey et al., 1993, Blair et al., 1997). Chymase may to be a potent inductor of neovascularization via VEGF (Muramatsu et al., 2000a), an angiogenic mediator that has been demonstrated to be in high concentrations both in lesions and in the peritoneal fluid of women affected by endometriosis (Donnez et al., 1998, Young et al., 2015).

High numbers of MCs has been reported to exist in endometriotic lesions (Matsuzaki et al., 1998a, Sugamata et al., 2005). Those studies only identified MCs using tryptase staining or toluidine blue dye. Missing from these results are any chymase positive cells and, by using the cationic dye on formalin fixed tissues they have also missed any MCs with a different composition of glycosilaminoglycans (strongly sulphated), which react differently with formalin fixation and so do not allow the cationic colour (toluidine blue) to enter the granules (Strobel et al., 1981, Wingren and Enerback, 1983). The current study is the first to have explored the MC phenotype in endometriotic lesions by considering both tryptase and chymase. Using double immunofluorescence MC_{TC} cells were detected in peritoneum and lesions of women with endometriosis. Remarkably, the majority of MCs present in the endometriotic lesions were identified to be of the MC_C phenotype, which would have been missed in previous studies. These findings suggest that the MCs in the lesions retain the

abnormal "endometrial" phenotype seen in these patients consistent with their origins being in endometriotic fragments that arrive in the peritoneum as a result of following after retrograde menstruation.

There is increasing evidence from conditions such as interstitial cystitis and irritable bowel syndrome, that mast cells play a critical role in the pathogenesis of chronic and neuropathic pain (Heron, 2013, Chatterjea and Martinov, 2015). MCs can release mediators that increase excitability of neurons, due to their capacity to store neurotransmitters such as substance P or nerve growth factor (NGF) in their cytoplasmic granules (Wernersson and Pejler, 2014). Due to the fact that chronic pelvic pain is a frequent and debilitating symptom in women affected by endometriosis, the current study set out to explore the phenotype of MCs in the peritoneal wall, a predominant location for endometriotic lesion growth, angiogenesis and nerve sprouting (Arnold et al., 2012).

Detailed analysis of human peritoneal biopsies conducted during this study has revealed that MCs are present in the peritoneal wall both in the recto-vaginal pocket (pouch of Douglas, "prone" site for endometriotic lesion formation) and in the frontal abdomen peritoneal wall ("distant" site from endometriotic lesion formation). This study is the first to report a substantial difference between samples from control and chronic pain/endometriosis patients. The pattern of immunoexpression of chymase in peritoneal sections was strikingly different between groups. Peritoneal MCs in control samples were MC_C and appeared highly activated throughout the section, both in prone and distant peritoneum with intense extracellular chymase staining. In contrast, immunofluorescence on tissues from women of grade 0 (pain/no lesions) or grade I-IV revealed a lower intensity of chymase staining, with the protease retained in the cytoplasm. Moreover, MCs appeared to have acquired a tryptase/chymase phenotype (MC_{TC}) in the peritoneum "prone" samples from endometriosis group biopsies, again pointing to plasticity in response to an altered peritoneal environment.

For the first time, this study has detected activated MC_C cells in the peritoneal wall. It is well known that the peritoneal serous membrane permits the passive dialysis of significant quantities of substances between the peritoneal fluid and the blood plasma (Young et al., 2013). The intense immuno-expression of chymase might be

resulting in increased capillary dilation via its conversion product, angiotensin II (Batenburg et al., 2004), contributing to fluid exchange. Further, angiotensin II is detectable in peritoneal fluid during the menstrual cycle under normal conditions (Delbaere et al., 1996). Notably, preliminary findings suggested chymase also present in mesothelial cells, the epithelial-like monolayer interface between peritoneal membrane and fluid. This result supports the potential role of chymase during blood-fluid dialysis.

It has been reported that mesothelium damage, increased angiogenesis together with fibrosis in peritoneal compartments may result in a decrease in the number of MCs (Jimenez-Heffernan et al., 2006, Stavenuiter et al., 2011). The reduction in chymase expression in the peritoneum of women suffering of endometriosis would appear consistent with MC adaptation to the altered endometriosis-related peritoneal environment (Burney and Giudice, 2012).

It is known that women affected by chronic pelvic pain and/or endometriosis show a central hyperalgesia (He et al., 2010). During the current study, both the number of MCs within the different groups and the concentrations of MC granule mediators in the peritoneal fluid were investigated. One potential source of mature MCs found in the peritoneal cavity could be the peritoneal fluid (Meurer et al., 2016). The current study established methods to quantifying the number of MCs in peritoneal fluid using flow cytometry. I found that the peritoneal fluid MCs represented a small portion of the total immune cell population, approximately 0.2% of live $CD45^+$ cells, and their numbers appeared to remain constant between the different groups (no pain, grade 0, grade I-II), with a small but non significant increase in the percentage in the moderate/severe endometriosis group, which needs to be investigated in a larger group of patients. After isolation of the immune cells, the peritoneal fluid was also assayed for proteases and histamine content; no change between groups was detected for tryptase or chymase but histamine concentrations were significantly lower in fluids from women with pain and more severe endometriosis. This finding appears consistent with an increased sensitivity to pain of women affected by chronic pain and/or endometriosis, as low concentrations of MC neurogenic mediators, tryptase and histamine, may still trigger the abnormal nociception.

To provide a further link between MCs and pain pathways, PAR-2 a receptor expressed on primary afferent neurons that can play a role in neuronal sensitization, which can be activated by tryptase was also investigated (Compton et al., 2001, Hoogerwerf et al., 2001, Noorbakhsh et al., 2003). For the first time, PAR-2 was detected in the human peritoneum. PAR-2 immuno-expression was higher in peritoneal samples collected during the secretory phase compared to proliferative phase, suggesting an influence of sex hormones on PAR-2 expression.

Notably, PAR-2 immuno-expression was reported to be of higher intensity in peritoneal biopsies from patients reporting chronic pelvic pain compared to controls whose were not attending the pain clinic. This observation may be explained by the different types of peritoneal biopsy, "prone" site corresponds to the cul-de-sac where the peritoneal fluid resides and is therefore, the primary location where effects of an altered environment would be detected. Remarkably, PAR-2 expression was much more intense in biopsies from women with pain and endometriosis and this was independent of the phase of menstrual cycle, and the location of the biopsy. Notably, PAR-2 immunoexpression was both increased and was detected in all the tissue samples, including the endometriotic lesions. Although, few MCs were localized within the sample tissue area. The findings brought new evidence consistent with neuronal sensitization in the peritoneum of women affected by endometriosis.

In this study, one of the significant limitations was the small sample size of 'no pain' control patients. This patient category is becoming increasingly difficult to reach as laparoscopic practise for sterilisation is not often performed in the United Kingdom nowadays. Moreover, endometriosis can often be asymptomatic, and half of the 'no pain' control samples investigated were in fact affected by endometriosis. It was also challenging to overcome to the high variability within human patients, since it was not always possible to reach statistical power with the tissue resources available. Moreover, the stratification of patients and exclusion criteria employed during this study meant a high number of samples were not used. With these observations in mind, further studies should be planned.

In summary, this is the first study to detect a different MC phenotype in eutopic endometrium from women affected by endometriosis, also present in the ectopic

lesions. The data also show that MCs are normally resident in the peritoneal wall, where they appear to be activated. In women with chronic pain and/or endometriosis, it appears that peritoneal wall MCs adapt their phenotype to the altered local micro-environment, acquiring a MC_{TC} phenotype. The presence of MCs in the peritoneal fluid was confirmed but their numbers and concentrations of tryptase and chymase did not change between cases of pelvic pain and/or endometriosis when compared to control samples. Women suffering from chronic pain and endometriosis present with a diffuse hyperalgesia, and the finding that PAR-2 protein was upregulated in these patient groups suggests a mechanism by which low concentrations of tryptase might still induce effects. Further studies are required to determine whether inhibition of PAR-2 might offer a therapeutic target in women with chronic pelvic pain.

These results have elucidated the possible role of MCs in the endometriotic lesion formation and the establishment of chronic pain, offer a concrete platform for further investigations in women and using model systems including cell cultures and mouse models.

Chapter 5 Exploring the use of *in vitro* and *in vivo* mast cell models

5.1 Introduction

Mast cells derive from $CD34^+/CD117^+$ pluripotent stem cells in the bone marrow. MC progenitors are recruited into peripheral tissue where they differentiate *in situ* (Metcalfe et al., 1997). The characteristics of mature human MCs are specific to the environment in which they mature and reside. MCs are present throughout the human body so they can vary considerably in morphology and granule content (Hoffmann, 2016).

MCs are also present in human reproductive tissues, and they are thought to be key regulators for implantation, menstruation and labour (Menzies et al., 2011). As detailed in Chapter 3, during normal physiology, MCs reside in both myometrial and endometrial uterine compartments. The activation of uterine MCs appears to be menstrual cycle phase dependent, and it is thus possible that MCs respond to steroid hormone fluctuations due to their expression of oestrogen and glucocorticoid receptors. Furthermore, MCs have been reported to be involved in endometriosis. In Chapter 4, extensive investigation has provided evidence of environment adaptation and a phenotype alteration in MCs in tissue biopsies from women affected by chronic pelvic pain and endometriosis. These findings set the scene for further exploration on MC behaviour in physiological and pathological conditions using both *in vitro* and *in vivo* models.

Few attempts have been made to isolate mature MCs from tissues, for example Kulka and Metcalfe (2001b) illustrated the isolation of mature human mast cells from skin biopsies, and Sperr et al. (1994) described the isolation of cardiac MCs. However, although it has been shown to be possible to isolate live mature MCs, issues may arise with both number and purity of the cells. MC phenotype can be also strongly influenced by the isolation techniques (Andersen et al., 2008). For this reason, both primary cultures of mast cells derived from stem cells and/or immortalised mast cell lines are typically used for the study of MC function *in vitro*.

Several studies on MC biology have been accomplished using *in vitro* maturation of MC progenitors, such as $CD34^+$ or $CD133^+$ cells, which are typically isolated from peripheral blood or umbilical cord blood (Rådinger et al., 2010). At the end of an 8-week *in vitro* differentiation protocol as described by Kirschenbaum and Metcalfe (2006), peripheral blood-derived MCs appeared to be relatively mature, with a condensed non-segmented nucleus and numerous toluidine blue positive granules. Moreover, these mature blood-derived MCs have a tryptase/chymase phenotype (MC_{TC}), in contrast to cord blood-derived MCs, which are relatively immature and chymase negative (Mitsui et al., 1993, Durand et al., 1994). Both PBMCs and CBMCs are functionally active; they can degranulate upon IgE-dependent and independent stimulation (Valent et al., 1992, Moon et al., 2003, Saini et al., 2009, Jensen et al., 2014).

However, because of the rarity of tissue MCs and the expense of the differentiation procedures used to mature, MC cell lines have been established and they are the most used *in vitro* models. The first MC line was derived by Butterfield et al (1988) who isolated MCs from a patient with MC leukaemia, and established the HMC-1 (Human Mast Cell-1) cell line. The HMC-1 was shown to have two point mutations in the c-kit receptor (Furitsu et al., 1993). The c-kit mutation means the HMC-1 cells remain in an undifferentiated state, with low granule content of tryptase and chymase (Nilsson et al., 1994a, Guhl et al., 2010).

The importance of MCs in the aetiology of disorders such as asthma, has been explored in *in vivo* models, predominantly in the genetically modified animals. The c-kit/SCF axis is vital for MC development, which has led to the extensive use of several strains of c-Kit/SCF mutant mice in MC research (Galli and Kitamura, 1987). The composite genotype $Kit^{W}/Kit^{W\text{-}v}$ causes profound mast cell deficiency (Kitamura et al., 1978), these animals are anaemic and also infertile. Thus, more widely used is a more recent MC deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ strain ("sash"), as these animals do not present some of the more critical disadvantages that characterize the $Kit^{W}/Kit^{W\text{-}v}$ strain (Lyon and Glenister, 1982). However, due to the important role of c-kit in the development of other immune cells, $Kit^{W\text{-}sh/W\text{-}sh}$ mice are also deficient in neutrophils and T cells (Katz and Austen, 2011) which can complicate the interpretation of the role of MCs. $Kit^{W\text{-}}$

sh/W-sh mice are described as "fertile"; however, "sash" colonies often present irregular birth rates and high natal and postnatal death rates. Further, $Kit^{W\text{-}sh/W\text{-}sh}$ female mice display severely impaired implantation, although single females presented normal litter sizes (Zenclussen and Hammerling, 2015).

Immune cells play important roles in reproduction (Salamonsen and Lathbury, 2000) and the role of MCs has been studied in both wild type and genetically modified animal models (Menzies et al., 2012). In mice, MCs have been identified using toluidine blue staining in the uterus. Toluidine blue positive cells appear to vary in number through the different phases of the oestrus cycle, showing a peak during oestrus (fertile phase) and remaining high during early gestation (Woidacki et al., 2013a, Woidacki et al., 2013b).

The models described above offer the potential to be useful tools to investigate the contribution of MCs to health and disease of the endometrium, including processes physiologically and pathologically regulated by circulating sex steroid hormones, such as endometriosis (Critchley and Saunders, 2009, Burney and Giudice, 2012). Thereby, during the current study, a mouse model that efficiently recapitulates the endometriotic condition was employed, where syngeneic menstrual endometrial tissue was introduced into the peritoneum of immunocompetent recipient mice (Greaves et al., 2014a). Whilst, MCs have been implicated in regulation of menstruation, due to their ability to secrete extracellular matrix proteases (Zhang et al., 1998), this role has not been explored using mouse models of menses, where key processes as tissue breakdown and remodelling are induced by administration of exogenous sex steroid hormones followed by progesterone withdrawal (Cousins et al., 2014).

To complement and extend the studies on human tissues described in Chapter 3 and 4, the studies in this chapter explored different methods currently available to investigate MC behaviours at baseline and under oestrogenic treatments using peripheral blood derived primary cells, the HMC-1 cell line and the in-house murine models of menstruation and endometriosis.

5.1.1 Summary

Mast cells are immune cells of the myeloid lineage, which are present in both mucosal and connective tissues throughout the body, including the endometrium. Their activation and degranulation notably regulates many aspects of physiological and pathological processes. Studies described elsewhere in this thesis have identified MCs in both normal uterine tissue and in endometriotic lesions and peritoneum. Notably, mature MCs expressed ERβ and thus MCs may be a target for oestrogens.

With these observations in mind, we hypothesised that MCs are able to respond to oestrogen action. The current investigation was designed to elucidate the phenotype of peripheral blood-derived MCs, the HMC-1 cell line and the mechanism of oestrogenic stimulation of the latter *in vitro*. Moreover, the potential role of MCs in murine models of menstruation and endometriosis was investigated for the first time.

5.2 Aims of the Chapter

1. To use *in vitro* cell model to determine whether the phenotype and function of MCs can be influenced by sex steroid hormones.
2. To use mouse models of menstruation and endometriosis to investigate putative roles of MCs in the physiology and pathology of the endometrium.

5.3 Material and methods

5.3.1 Peripheral blood derived-mast cell culture

Peripheral blood, bone marrow and umbilical cord blood are the most common sources of $CD34^+$ progenitor cells from which to differentiate MCs (Welker et al., 2000, Kinoshita et al., 1999). For this study, we decided to isolate MC progenitors from peripheral blood under the LREC 08/S1103/38, held by MRC Centre for Inflammation Research, University of Edinburgh.

5.3.2 Preparation of peripheral blood for $CD34^+$ selection

$CD34^+$ pluripotent progenitor cells were collected from peripheral blood from female donors (24-47 age range), n=20. On average, peripheral blood contains 0.01–0.1% $CD34^+$ cells.

Briefly, 50ml-polypropylene Falcon tubes (Fisher Scientific, UK) were preloaded with 4ml of sodium citrate (3.8% in H_2O, Sigma Aldrich, UK). Samples (40ml to 80ml) of venipuncture-derived peripheral blood were collected from healthy donors. Each 10ml of peripheral blood and anti-coagulant solution was mixed with 25ml of complete StemPro serum-free medium with nutrient supplement (Gibco, UK), 2 mM L-glutamine, 100 IU/ml penicillin, and 50 μg/ml streptomycin (Life technologies, UK). This solution was layered onto 14ml of lymphocyte separation media (Histapaque, Sigma Aldrich, UK) and centrifuged at 675g for 20 minutes at room temperature. Following centrifugation, the red blood cells formed a layer below the separation media at the bottom of the tube, while mononuclear cells (cells of interest) were identified above the layer of separation media. Media above this layer was removed carefully before recovery of the monolayer cells. These recovered mononuclear cells were mixed with 25ml of complete StemPro media and centrifuged at 300g for 10 minutes to remove any debris. Following centrifugation, the supernatant was removed and the pellet of mononuclear cells resuspended in 25ml of StemPro serum-free complete medium. The aforementioned step was repeated twice. The final cell pellet was re-suspended in 1x phosphate buffered saline, pH 7.2 (PBS, Gibco, UK), containing 0.5% bovine serum albumin (BSA) (Sigma Aldrich, UK) and 2mM ethylene diamine tetra-acetic acid (EDTA, Gibco, UK) and cells were counted using a haemocytometer (Immune Systems, UK).

5.3.3 Magnetic labelling and magnetic separation

The $CD34^+$ MicroBead Kit contains MicroBeads conjugated to CD34 antibodies for magnetic isolation of CD34-expressing cells from peripheral blood. In this study cells were labelled with the $CD34^+$ MicroBead Kit from MACS Miltenyi Biotec, UK, which is designed to allow very high levels of $CD34^+$ purity.

The purified white blood cell suspension was centrifuged at 300g for 10 minutes and the supernatant removed and the pellet re-suspended in 300 µl of PBS-EDTA buffer (1x phosphate buffered saline (PBS) + 0.5% BSA + 2mM EDTA) and mixed with (FcR) Blocking Reagent and CD34+ MicroBeads (100µl each) and incubated for 30 minutes at 4°C. After incubation, cells were washed with 300 µl PBS-EDTA buffer and centrifuged at 300g for 10 minutes. The supernatant was removed and the cells were re-suspended in 500µl of buffer. Magnetic separation was performed according manufacturer's protocol using a mini MACS separator (Miltenyi Biotec, UK). The final cell number was recorded using a haemocytometer (Immune Systems, UK).

5.3.4 Differentiation protocol

The differentiation protocol of PBMCs employed during this study was described by Kirschenbaum and Metcalfe (2006). In detail, isolated $CD34^+$ cells were cultured in serum-free complete culture media (Stem-Pro34® SFM, Gibco, Paisley, UK) containing recombinant human stem cell factor (rhSCF), recombinant human interleukin 6 (rhIL-6), and recombinant human interleukin 3 (rhIL-3, supplemented to the media only for first week) (Figure 5-1-A); rhSCF, rhIL-6 and rhIL-3 purchased from Peprotech, London, UK). Hemidepletion was performed weekly, by removing adherent cells and debris. The cell suspension, containing differentiating mast cells, was centrifuged at 150g for 5 minutes. The cell pellet was then resuspended in 25ml of fresh complete StemPro media with rhIL-6 and rhSCF. Differentiation of MCs within the cultures was assessed weekly using acidic toluidine blue staining (TB: 0.1% toluidine blue, Scientific Laboratory Supplies, East Riding, UK; in 0.5N HCl, Sigma Aldrich, Dorset, UK). Cells were isolated using a cytospin method at 300rpm for 3 minutes (Cytospin2, Shandon, ThermoScientific, Paisley, UK). They were subsequently fixed in Carnoy's for 1 hour (60% absolute alcohol, VWR Prolabo, UK,

30% chloroform and 10% acetic acid, Sigma Aldrich, UK), incubated in 70% ethanol and then stained in acidic toluidine blue for 45 minutes before air-drying overnight. Slides underwent 95% alcohol dehydration before mounting with Permafluor (ThermoFisher Scientific, UK). A complete differentiation (95-100% of mature MCs, Figure 5-1-B) was achieved over a 8‑10-week culture period (Kirshenbaum and Metcalfe, 2006).

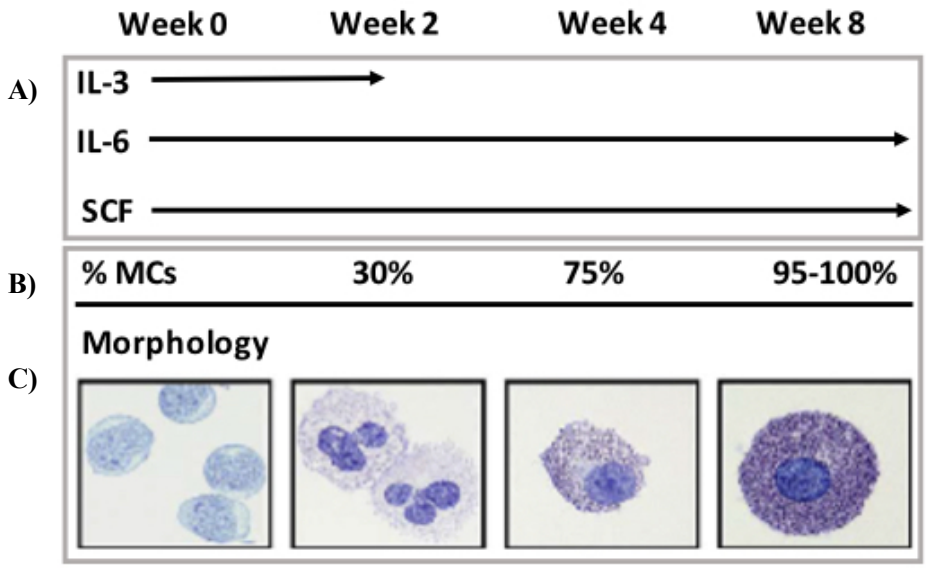

Figure 5-1 Summary of protocol used to derive human mast cells derived from CD34⁺ peripheral blood progenitors
A) Weekly interleukins addition, **B)** Percentage of expected mast cells, **C)** Toluidine blue staining for morphology assessment. Image adapted from Kirshenbaum and Metcalfe (2006).

5.3.5 Human mast cell (HMC-1) cell culture

The human mast cell line, HMC-1, was originally established in 1988 using cells recovered from the peripheral blood of a patient with mast cell leukaemia (Butterfield et al., 1988). This cell line has been used extensively for *in vitro* studies of human mast cell behaviour (Kitayama et al., 1993, Nilsson et al., 1994a). An aliquot of HMC-1 cells was generously gifted by Dr Butterfield allowing me to conduct *in vitro* mast cell investigations described in this chapter.

HMC-1 cells were cultured at a density of $4x10^5$ cells/ml in Iscove's Medium containing phenol red as a pH indicator (IMDM, with 25mM 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3.024g/l sodium bicarbonate buffer, 4mM L-glutamine; ThermoFisher Scientific, UK) with 10% iron-supplemented calf serum

(Hyclone, USA), 1.2mM α-thioglicerol (Sigma Aldrich, UK) and 10ml/l penicillin/streptomycin (10,000 units of penicillin and 10 mg streptomycin per every 10ml of culture media, (Sigma Aldrich, UK). Cells were incubated at 37°C with 5% CO_2 (Heraerus, HERACell 150, ThermoFisher Scientific, UK). HMC-1 cells were seeded at an optimal concentration of $4x10^5$ cells/ml for cell expansion once a week.

5.3.6 Oestradiol treatment

To avoid exposure to oestrogens during culture, HMC-1 cells were seeded at 10^5 cells/ml in phenol red–free complete (white) Iscove's Medium (ThermoFisher Scientific, UK) containing steroid-stripped foetal calf serum (FCS, Life Technology, UK) and 10,000 units of penicillin and 10 mg streptomycin per every 10ml of culture media, Sigma Aldrich, UK). After 72 hours in this white "complete" medium, cells were collected, centrifuged for 5 minutes at 800 rpm, acceleration and brake 9/10 (MSE Centrifuges, Mistral 3000i, UK) at room temperature. The supernatant was discarded and the cell pellet resuspended in a small volume (approximately 200µl) of serum free white IMDM with antibiotics (as above); 10µl of this cell suspension were mixed 1:1 with trypan blue (Invitrogen, UK), and the HMC-1 cells were counted with the Countessa ™ (Invitrogen, UK). After counting, cells were seeded at 10^5 cells/ml in serum-free IMDM for 16h before treatments.

17β-oestradiol and fulvestrant (E_2: Sigma Aldrich, UK; fulvestrant or ICI: Tocris, UK) were dissolved into dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) to form stock concentrations of 10^{-2} M. Working stock solutions were made in sterile PBS without Ca^{2+} and Mg^{2+} (Invitrogen, UK) to the 10^{-4} M for E_2 and 10^{-5} M for ICI. Final concentrations (in well) used were at 10^{-7} M and 10^{-8} M for E_2 and 10^{-6} M ICI. Cells were pre-treated for 1 hour with ICI 10^{-6} M before conducting the experiment, for allowing full antagonism for both ER isoforms (Osborne et al., 2004).

5.3.7 RNA extraction from primary cells and cell lines

The differentiating $CD34^+$ progenitor cells and HMC-1 cell line used in this study were grown in suspension (differentiation protocol for $CD34^+$ progenitor cells: Section 5.3.4 and HMC-1 cell line: Section 5.3.5), cultures were centrifuged for 5 minutes at 800rpm (MSE Centrifuges, Mistral 3000i, UK) at RT before proceeding

with RNA extraction. The conditioned media was removed and cells were resuspended and lysed with 350µl PureLink Lysis buffer with RNA carrier and 1% β-mercaptoethanol (Sigma-Aldrich, UK) for primary cells (concentration $<10^6$ cells) and with 350µl RNase Lysis Buffer (RLT, Qiagen, UK) with 1% β-mercaptoethanol for samples with $>10^6$ cells. For homogenization, the cell lysate was transferred into a QIAshredder spin column (Qiagen, UK). QIAshredder columns were centrifuged for 2 minutes at 12,000g at RT. An equal volume (350µl) of 70% ethanol was added to each sample, the solution from HMC-1 samples was then pipetted into a RNeasy spin column and the solution from primary differentiated mast cells was added to a PureLink spin column. RNA extraction protocol was carried out by following the steps described in Section 2.2.1.

5.3.8 Preparation of cDNA

Reverse transcription of RNA was performed as described in Section 2.3.

5.3.9 Quantitative Real Time PCR (Taqman® method)

The Taqman® method of polymerase chain reaction (PCR) was used for this study, according to principles and reagents in Section 2.4; probes and primers are detailed in Table 2.2.

5.3.9.1 Relative mRNA expression and statistical analysis

PCR analysis was conducted by using the standard curve method (Section 2.4.2) and compared to concentrations of mRNAs in week1 undifferentiated $CD34^+$ mRNA for primary cells or the Ishikawa cell line (European Collection of Cell Culture, ECACC n.99040201, UK) as control mRNA for HMC-1.

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, USA). Data are presented as mean ± standard error. For two group analysis, an unpaired two-tailed t-test was used, with Mann-Whitney post-test. One-way ANOVA was further used for multiple group comparison, followed by Kruskal-Wallis as a secondary test and Dunn's multiple comparisons test. Criterion for significance was $p<0.05$.

5.3.10 Separation of DNA in polyacrylamide gels

DNA separation was carried out using polyacrylamide gels as described in Section 2.4.3.

5.3.11 Protein extraction from primary blood-derived mast cells

Proteins were extracted from PBMCs following the application of the differentiation protocol, in Section 5.3.4. All cells (concentration was not assessed due to limited cell number at the end of differentiation protocol) were collected into a 1.5 ml microcentrifuge tube and centrifuged at 800rpm for 5 minutes at RT (MSE Centrifuges, Mistral 3000i, UK). Cell media was discarded and the cell pellet was washed with ice cold PBS. After centrifugation cells were lysed using 350µl of our laboratory adapted RIPA buffer (Table 5-1). The resulting lysate was vortexed and centrifuged for 10 minutes at 13,000g at 4°C to eliminate cellular debris. The supernatant containing total protein extract was transferred to a fresh pre-chilled 1.5ml microcentrifuge tube and stored at -80°C until further use.

Reagents – Stock concentration	Final concentration
Tris pH 7.5 1M (Sigma-Aldrich, 252859)	50mM
EDTA pH 8.5 0.5M (Sigma-Aldrich, E5134-500g)	5mM
NaCl 1M (Sigma-Aldrich, S3014-500G)	150mM
Triton-X (Sigma-Aldrich, T8787)	1%
Aprotinin (protease inhibitor; Sigma-Aldrich, 10981532001)	2µg/ml
Halt Protease Inhibitor Cocktail (x100; ThermoFisher Scientific, 87786)	1x

Table 5-1 Saunders laboratory adapted RIPA buffer for protein extraction.

5.3.12 Extraction of nuclear and cytoplasmic fractions from the human mast cell-cell line (HMC-1)

In this study, some proteins of interest were present in the nuclei; therefore separation of the nuclear and cytoplasmic fractions was undertaken for a more robust quantification. Nuclear extraction was performed using the Active Motif kit (40010, Active Motif, La Hulpe, Belgium). The manufacturer's instructions were followed throughout the protocol; buffers are as described in Table 5-2.

For preparation of nuclear extracts, $8.8x10^6$ cells were resuspended in 3ml ice cold PBS containing phosphatase inhibitors (Table 5-2). Samples were centrifuged for

5 minutes at 200g at 4°C. The supernatant was discarded and the cell pellet was gently resuspended in 500µl of 1x hypotonic buffer (Table 5-2), transferred into a pre-chilled 1.5ml microcentrifuge tube and kept on ice for 15 minutes. The addition of hypotonic buffer together with 25µl of detergent caused the cell membranes to swell thereby releasing cytoplasmic protein into the supernatant. The samples were then centrifuged at 14,000g for 30 seconds at 4°C, to separate the intact nuclei from the ruptured cytoplasmic membranes and the cytoplasmic proteins. Supernatant containing the cytoplasmic fraction was transferred and stored at -80°C until further use. The nuclear pellet was resuspended in 50µl of complete lysis buffer (Table 5-2) with 2.5µl of Active Motif detergent and mixed by vigorously pipetting up and down to disrupt cell nuclei and facilitate solubilisation of membrane-associated nuclear proteins. Due to the formation of a very viscous pellet, high speed vortexing for 10 seconds was required before proceeding to the following step. The nuclear suspension was incubated on wet ice for 30 minutes on a rocking platform set at 3g/min (Heidolph Polymax 2040, Schwabach, Germany). Samples were vigorously vortexed then centrifuged at 14,000g for 10 minutes at 4°C, to separate nuclear proteins from nuclear membrane residues. The resultant nuclear fraction was stored at -80°C prior to further analysis.

Reagent	Components	Volume needed for $8.8x10^6$ cells
PBS/Phosphatase Inhibitors	10x PBS	0.8 ml
	Distilled water	6.8 ml
	Phosphatase Inhibitors	0.4 ml
	Volume required	8 ml
1x Hypotonic buffer	10x Hypotonic buffer	50 µl
	Distilled water	450 µl
	Volume required	500 µl
Complete lysis buffer	10 nM DTT	5 µl
	Lysis Buffer AMI	44.5 µl
	Protease Inhibitor Cocktail	0.5 µl
	Volume required	50 µl
Detergent	Volume required	27.5 µl

Table 5-2 Active Motif buffers recipes used for nuclear and cytoplasmic fraction extraction.

5.3.13 Western Blotting

Western blotting (WB) is an analytical technique used to quantify proteins in biological samples and it is dependent on the specificity of the interaction between the protein of interest and an antibody. Western blotting relies on specific antibodies binding to proteins that have been separated based on size by gel electrophoresis and

then transferred onto a membrane made of nitrocellulose or polyvinylidene fluoride (PVDF). In this study, PVDF membranes were used. After electrophoresis, the gel was placed directly onto the membrane and proteins were transferred from the gel onto the membrane by application of an electrical current. The membrane was then processed as described in details in section 5.3.13.1.

Protein samples were placed on ice, before mixing with NuPage® LDS sample buffer (ThermoFisher Scientific, UK) and the NuPage® reducing agent (ThermoFisher Scientific, UK); concentrations and the final volume are detailed in Table 5-3. Sample preparation is used to break protein disulphide proteins bonds releasing secondary structure and maintaining the proteins in a denatured state for electrophoresis. Lithium dodecyl sulphate (LDS), present in the sample buffer, together with DTT (reducing agent), facilitates protein bond breakage. Samples were heat-denatured at 70°C for 10 minutes on a hot dry block and placed on ice until loading into individual wells on the polyacrylamide gel.

Reagents	Final Concentration/Volume
NuPage LDS sample buffer (4x)	1x
NuPage reducing agent (10x)	1x
Deionized water	Based on protein volume
Protein sample	30-40µg per well
Total volume	37µl – 10 well comb, 1mm thick gel 25µl – 15 well comb, , 1mm thick gel

Table 5-3 Protein samples preparation recipe for NuPage® gel loading.

NuPage® Novex Bis-Tris Precast polyacrylamide gels (4%-12%; Life Technologies, UK) were fitted in a XCell *SureLock*™ Mini-Cell (ThermoFisher Scientific, UK) gel tank, filled with 1X NuPAGE® MOPS SDS Running Buffer (ThermoFisher Scientific, UK). NuPAGE Antioxidant (500µl, ThermoFisher Scientific, UK) was added to the running buffer in the upper (cathode) chamber allowing the antioxidant to migrate with reduced proteins, to maintain reducing conditions and prevent reoxidation of amino acids.

The protein samples were added (volume dependent on gel type, as described in Table 5-3) to the wells together with the tracking dye, the Chameleon SeeBlue® Plus 2 pre-stained molecular weight markers (Life Technologies, LC5925, UK) using

gel loading pipette tips (Alpha Laboratories, GL20037NTRS, UK). The gel tank was run at 200V for 50 minutes with an expected current between 60-70mA.

5.3.13.1 Semi-dry electrophoretic protein transfer

Once electrophoresis was complete, the gels were transferred using the semi-dry electrophoretic transfer method. Firstly, the gel was equilibrated in 1x Transfer Buffer (Table 5-4) for 15 minutes to remove electrophoresis buffer salts and detergents. The Immobilon®-FL membrane (Millipore IPFL00010, UK) was pre-cut to the dimensions of the gel and soaked in methanol for 15 seconds for equilibration. The membrane used during this study, was made of polyvinylidene fluoride (PVDF). PVDF membranes have very low auto-fluorescence across a wide range of excitation/emission wavelengths, making then ideal for fluorescence-based immune detection.

Six sheets of Whatman 3 mm Chr filter paper (Sigma Aldrich, UK) per gel together with the membrane were soaked in 1x Transfer buffer for 5 minutes (recipe in Table 5-4). Three sheets of Whatman paper were placed on the anode plate of a Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad 1703940, UK), followed by the membrane and three filter papers. Air bubbles were removed before performing the transfer. The semi-dry transfer took place at 14V for 90 minutes.

Western Transfer Buffer (10X)		
Tris	30.28g	250mM
Glycine	144g	1.92M
SDS	1g	0.1%
Distilled water added to 1 litre. For 1x 20% methanol was added		

Table 5-4 Western Blot Transfer buffer composition

5.3.13.2 Antigen blocking and antibody incubation

The membrane was washed three times with 1x PBS and blocked to avoid non-specific binding of the antibodies, after transfer was completed. The Odyssey blocking buffer (at 1:1 dilution in 1xPBS, LiCor P/N 928-40000, UK) was used to reduce non-specific binding as it contains a mix of non-mammalian proteins dissolved in Tris. The membrane was incubated with the aforementioned blocking buffer for one hour at room temperature on a shaker. The membrane was then incubated with the primary antibody diluted in the blocking buffer, see Table 5-4, with the addition of 0.1%

Tween-20 overnight at 4°C on a shaker. For quantification purposes, the addition of a second primary antibody that recognises an endogenous protein control was required. Primary antibodies used for WB studies are illustrated in Table 5-5.

Antibody	Species	Clone and Supplier	Concentration	Dilution used
ERα	Mouse	ER6F11 - Vector Laboratories	7.5mg/ml	1:20
ERβ	Mouse	PPG5/10 - AbSerotec	1.0mg/ml	1:20
PCNA	Rabbit	FL-261 - Santacruz	20mg/ml	1:500
β-Actin	Rabbit	Abcam	10mg/ml	1:500

Table 5-5 Primary antibodies used for detection of human cell extracts during Western Blotting protocol.

5.3.13.3 Secondary antibody detection

After primary antibody incubation, the membrane was washed three times with 0.1% Tween-20 in PBS at room temperature on a shaker followed by incubation with the secondary antibody diluted in blocking buffer + 0.1% Tween-20 for 1 hour at RT on a shaker. The secondary antibodies used (Infra-Red Dye secondary antibodies, LiCor Biotechnology, UK) were conjugated with a fluorescent dye as illustrated in Table 5-6.

Antibody	Primary antibody to detect	Concentration	Dilution	IRDye	Colour of emission
Donkey anti-mouse	ERα ERβ Tryptase	7.5mg/ml 1.0mg/ml 1.0mg/ml	1:10000	800CW	Red
Donkey anti-rabbit	PCNA β-Actin	20mg/ml 10mg/ml	1:10000	680CW	Green

Table 5-6 Conjugated secondary antibodies used during Western blotting.

Two secondary antibodies were added at the same time to the buffer; to enable detection of both the protein of interest and the endogenous control protein. They were raised in different species and conjugated to different fluorochromes with separate excitation and emission spectra to avoid cross-reactivity. The membrane was washed four times with 0.1% Tween-20 in PBS at RT on a shaker. It was covered in 1xPBS to prevent it drying out before performing the scan with a LiCor detection system (LiCor, Odyssey CLx, UK).

5.3.14 Immunocytofluorescence

Protein localization in cells cultured *in vitro* was achieved by fluorescent immunocytochemistry. Cells from the human mast cell line (HMC-1, Section 5.3.5)

were centrifuged at a concentration of 10^4 cells/100µl onto uncharged slides (VWR Collection, UK) at 300 rpm for 3 minutes using a cytospin centrifuge (Cytospin2, Shandon, ThermoScientific, UK). Slides were washed in 1x PBS and cells were fixed with ice cold methanol (VWR Prolabo, UK) for 20 minutes at -20°C. Endogenous peroxidase was blocked with methanol containing 0.15% H_2O_2 for 15 minutes at RT. Slides were then washed with tap water followed by TBS for 5 minutes each. Detection of cytoplasmic or nuclear antigens required a permeabilisation step, which was accomplished by using a permeabilisation solution comprised of 0.2% Triton-X (Sigma Aldrich, UK), 5% NGS and 1% BSA in 1x PBS. The permeabilisation solution was applied for 20 minutes at RT in a humidity chamber. The same solution was used as the diluent for primary antibodies which were incubated overnight at 4°C.. The following steps for detection of protein are as described in immunofluorescence sections 2.5.11.

5.3.15 Enzyme-linked Immunosorbent Assay

5.3.15.1 Human tryptase ELISA

The non-competitive ELISA, SEB070Hu for tryptase (Cloud-Clone Corp., USA) was used for quantifying levels of tryptase in the culture medium of HMC-1. Details of the protocol are described in Chapter 2, Section 2.6.

5.3.15.2 Mouse tryptase (MCP-6) and chymase (MCP-5) ELISAs

Non-competitive "sandwich" ELISA kits were also used for animal studies: CSB-EL024128MO for tryptase beta-2 and CSB-EL005599MO for chymase (Cusabio Life Science, China). Standards were respectively: tryptase 5000pg/ml to 78pg/ml (5000, 2500, 1250, 625, 312, 156, 78 pg/ml) and chymase 10 ng/ml to 0.56 ng/ml (10, 5, 2.5, 1.25, 0.625,0.312, 0.156 ng/ml).

The protocol was performed by following manufacturer's instructions. Capture antibodies were pre-coated onto microplates. Standards, samples and blank were pipetted into the wells; plates were incubated for 2 hours at 37°C. After removing any unbound substances, a biotin-conjugated antibody specific for tryptase or chymase was added to the wells (100µl of 1x biotin-antibody) and incubated for 1 hour at 37°C. After washing (3x with ELISA kit washing buffer), 100µl of avidin conjugated-HRP was added to the wells, and the plate was re-incubated at 37°C for 1 hour. Following

3 washes to remove any unbound avidin-enzyme reagent, 90µl of TMB substrate solution was added to each well and colour developed in proportion to the amount of tryptase or chymase bound in the initial step. The colour development was stopped after 15-30 minutes by adding 50µl of stop solution to each well and the intensity of the colour is measured, as described in Section 4.3.9.1.

5.4 Studies using mouse models

All animal procedures were performed according to the Home Office Animals (Scientific procedures) Act 1986, under Professor Saunders project licence number PPL 60/4208 and under the personal licence number PIL I3FD3D49F.

Wild type C57BL/6J mice were either supplied by the University of Edinburgh Central Biological Services from in-house breeding stock or they were purchased from Charles Rivers Laboratories (Charles Rivers Laboratories International, UK).

Mice were housed in standard conditions, with food and water *ad libitum* and a light/dark cycle of 12 hours, under controlled temperature.

5.4.1 Collection of mouse tissues and peritoneal lavage

Cycling female C57BL/6J and animals undergoing the menstruation or endometriosis model protocols (Section 5.4.3 and 5.4.4) were sacrificed by asphyxiation using increasing levels of carbon dioxide (CO_2). Uterine horns and peritonea were dissected and fixed in Carnoy's for 6 hours before being embedded in paraffin, sectioned and used to localize MCs using toluidine blue staining.

5.4.2 Oestrus cycle assessment in cycling animals

Uteri from adult female mice were collected for spatial and temporal evaluation of MC numbers. Female C57BL/6J mice were sacrificed by asphyxiation using increasing levels of carbon dioxide (CO_2); 1ml PBS flushes of the vaginal secretions were carried out on each mouse and pipetted onto individual uncharged glass slides and allowed to air-dry overnight. Vaginal cells were fixed in ice cold methanol for 20 minutes, placed in Harris' haematoxylin for 30 seconds, in Scott's tap water for 30 seconds and eosin for 2 seconds, washed in tap water between the steps.

The stage of the cycle was determined using a bright field microscope by two independent observers. The four stages of the oestrus cycle are characterised by the presence or absence of immune cells and the shape of the epithelial cells as shown in Figure 5-2. At the time of cull, uterine horns were dissected and fixed in Carnoy's for 6 hours before paraffin embedding followed by toluidine blue staining (Section 5.4.5).

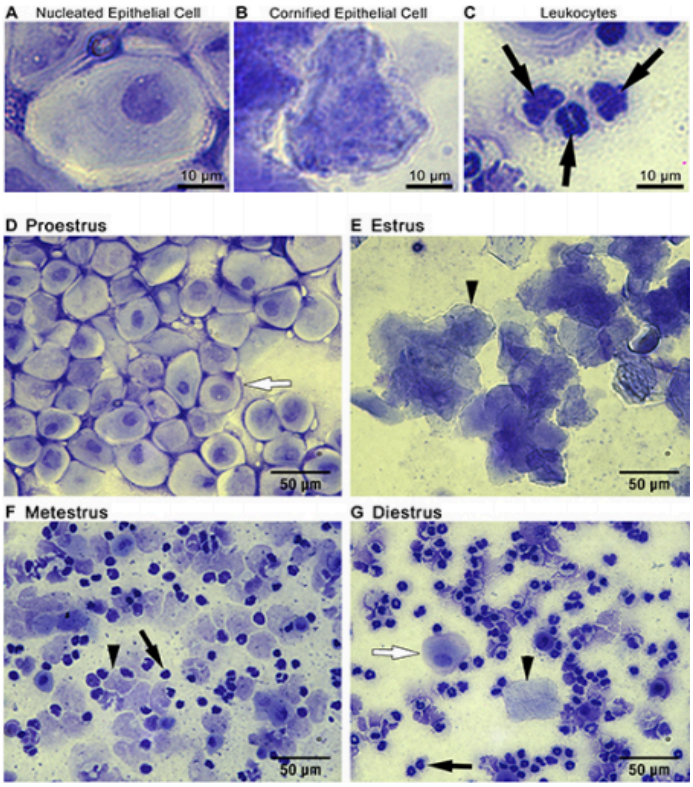

Figure 5-2 Cytological appearance of vaginal smears during different phases of the oestrus cycle.

A-B-C) Images represent the visual tool to assess stage of oestrus cycle in mice. **A-B)** show the different nuclear and cellular shape of epithelial cells. **C)** illustrates the presence of immune cells, such leukocytes. During the first phase, the proestrus **(D)** cells present in the smear are mainly rounded and nucleated epithelial cells, which transformed into cornified squamous cells during oestrus **(E).** Influx of immune cells starts during metestrus phase **(F)** and the ratio of immune cells to epithelial cells increases during diestrus **(G).** Black arrows indicate leukocytes, white arrows point nucleated epithelial cells, and black arrowheads show cornified squamous epithelial cells. Images taken from McLean et al. (2012).

5.4.3 Mouse model of menstruation

The mouse model of menstruation used during this study followed the protocol adapted in house and published by Cousins et al. (2014). In this study uterine tissues were retrieved from mice 24h and 48h after removal of progesterone pellets. A summary of the protocol is shown in Figure 5-3 and it is described in detail in the following sections.

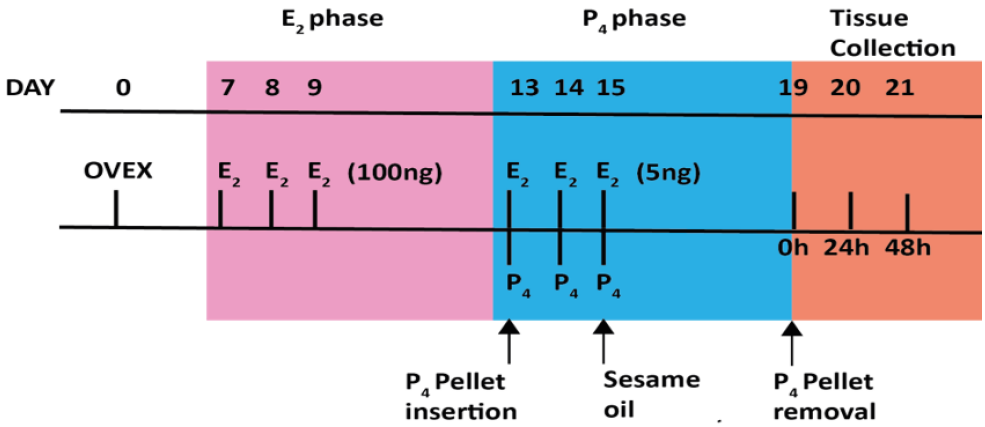

Figure 5-3 Overview of the Edinburgh mouse model of menstruation (Cousins et al., 2014). Endogenous ovarian hormones were reduced by bilateral ovariectomy at day 0. After 7 days of recovery, animals were injected with E_2, to mimic the E_2 dominated human proliferative phase (E_2 at 100ng/100µl dose was repeated daily, for a total of 3 days, until day 9). At day 13, to mimic the human secretory phase, animals reduced injections of E_2 (5ng/100µl daily, for 3 days) and a silastic mini pump of P_4 was inserted under the skin at the neck site. Decidualisation was induced by a mechanical stimulus, 20µl sesame oil delivery into the uterine lumen via cervix. P_4 withdrawal was induced by P_4 pellet removal at day 19. Samples were then collected 24h and 48h after P_4 withdrawal (day 20 and 21, respectively). OVEX: ovariectomy, E_2: 17β-oestradiol, P_4: progesterone. The 17β-oestradiol concentrations are reported in brackets.

5.4.3.1 Preparation of oestrogen for subcutaneous injection

Two E_2 stock solutions were prepared; a 1mg/ml stock solution and a 50µg/ml stock solution, using crystallised E_2 (E8875, Sigma-Aldrich, UK) and absolute ethanol (VWR Prolabo, UK). Final working solutions of 1µg/ml and 50ng/ml were prepared using a 1:1000 dilution of stock solutions in sesame seed oil (S3547, Sigma Aldrich, UK). Solutions were protected from light and left on the roller overnight at RT to allow complete solubilisation of E_2.

5.4.3.2 Preparation of progesterone silastic pellets

Rubber tubing (Silastic tubing Cat #508-008, 1.57mm I.D x 3.18mm OD, Dow Corning, UK) was washed in 70% ethanol and rinsed in distilled water, air dried before cutting into 1.5 cm pieces. One end of the 1.5cm tubes was sealed using adhesive sealant (Dow Corning, UK), and then filled with crystalline P_4 (Sigma Aldrich, Dorset, UK) and sealed on the other side.

P_4 pellets were equilibrated for 48h prior to insertion, by soaking them in 1% charcoal stripped foetal calf serum (CSFCS, ThermoFisher Scientific, UK) in PBS at 37°C.

5.4.3.3 Mouse model of menstruation protocol (Figure 5-3)

Briefly at start of the protocol (day 0), 8-10 week old female C57BL/6J mice were ovariectomised by dorsal bilateral ovariectomy under isofluorane-induced anaesthesia (Isofluorane-Vet™, Merial Animal Health, UK). Mice received an injection of 0.1ml of sterile saline and a dose of the analgesic, buprenorphine (Vetergesic, 0.1mg/kg, Alstoe Animal Health, UK) for pain management at the time of surgery. A 7-day recovery period was needed to complete depletion of endogenous ovarian derived hormones before starting the protocol. On days 7, 8 and 9, mice received sub-cutaneous injections (0.1ml) of β-oestradiol (E_2) in sesame seed oil (1μg/ml, oestradiol and sesame oil purchased by Sigma Aldrich, UK), resulting in a final dose of 100ng/mouse. This stimulated cell proliferation and simulated the human proliferative phase. At day 13 of the protocol, mice were anaesthetised under isofluorane and progesterone (P_4) secreting pellets were inserted sub-cutaneously (neck site), to simulate secretion of progesterone by the corpus luteum in women following ovulation. On days 13, 14 and 15 mice also received sub-cutaneous injections (0.1ml) of E_2 in sesame seed oil (50ng/ml) daily, resulting in a final dose of 5ng/mouse. To induce endometrial decidualisation on day 15, mice were anaesthetised as above and a single embryo transfer device (NSets, Paratech, USA) was used to inject 20μl of sesame seed oil trans-vaginally via the cervix into the uterine lumen of one horn. The contra-lateral horn remained untreated and was used as an internal control.

P_4 withdrawal was induced by progesterone pellet removal at 90 hours after oil injection (morning of day 19). Animals were culled by asphyxiation using increasing levels of CO_2 gas at 24h or 48h after removal of P_4 pellet (day 20-21). Uteri were collected, separated from the surrounding fat layer and then weighed. Weight was used to complement visual inspection for assessing whether the decidualisation response had been robust. Tissues were placed in Carnoy's for 6-hour fixation prior to paraffin embedding according to standard protocols.

5.4.4 Mouse model of endometriosis

The mouse model of endometriosis used during this study followed the protocol established in house and published by Greaves et al. (2014a). Dr Greaves generously shared tissue samples she had generated using the mouse model to reduce the number of mice used during these studies. Briefly (Figure 5-4), menstruation was induced in adult donor mice (approximately 8 weeks of age) using the protocol detailed above (Section 5.4.3). The decidualized horn was recovered from donor mice culled on day 19, 4-6 hours after P_4 withdrawal (induced by pellet removal). Endometrial tissue was collected by scraping it away from the myometrium using a scalpel.

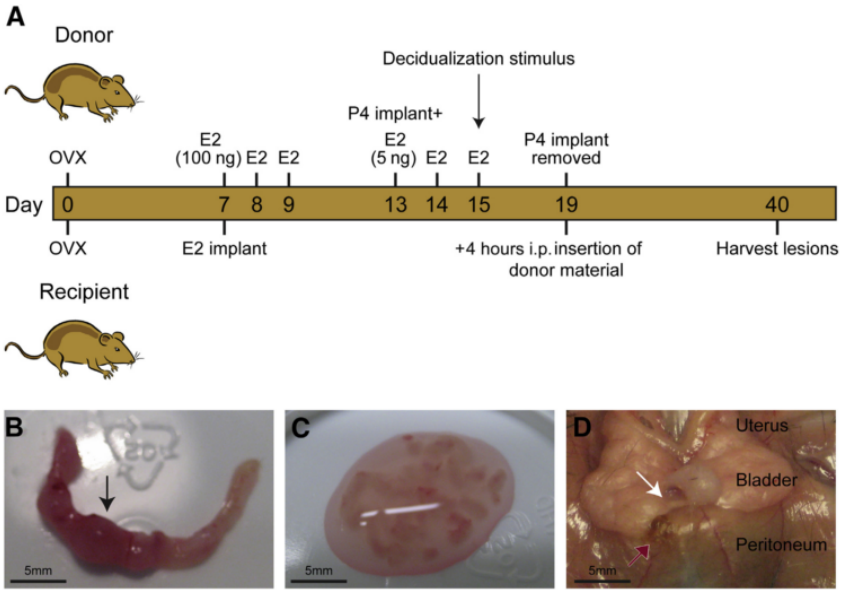

Figure 5-4 Endometriosis model timeline, protocol for donor and recipient females.
(A) Overview of the procotol that both donor and recipient animals underwent. For the donor group, it is illustrated above of the bar and followed the previous protocol. On day 19, donor animals were sacrificed 4-6 hours after P_4 removal and "donor" endometrial tissue was collected for i.p. injection into recipients. Recipients underwent ovx at the same time as donors, an E_2 implant was inserted at day 7, and they received by i.p. injection the decidualized material at day 19. **(B)** Decidualized uterine donor material, **(C)** endometrial tissue suspension for intraperitoneal injection **(D)** and location of endometriotic lesions at the time of tissue harvest. Image taken from Greaves et al. (2014a).

The tissue mass was prepared for intra-peritoneal injection by suspension in 200µl of sterile PBS (ThermoFisher Scientific, UK) and homogenised through a 19-gauge needle (BD Scientific, UK). This tissue suspension was then injected into anesthetized recipient mice (6-8 weeks of age). Recipient animals were ovariectomized at the same time as the donor, and they received an E_2 implant (Sigma Aldrich, UK; silastic implant: Dow Corning, UK) on day 7 of the protocol. The E_2

implant was prepared in the same way as the P_4 pellet, described in Section 5.4.3.2. Three weeks after i.p. injection, recipient mice were sacrificed. Peritoneal lavages were performed by injecting 2ml of sterile PBS into the peritoneal cavity to collect immune cells before tissue dissection. The uterine horns and peritoneal wall were fixed in Carnoy's for 6 hours, before performing toluidine blue staining. Peritoneal lavages were kept at 4°C, centrifuged at 900g for 10 minutes at 4°C and the supernatant solution was pipetted off and stored at -80°C for analysis by ELISA. Cell pellets were incubated in the dark with 5ml of 1x diluted RBC buffer for a maximum of 15 minutes (BioLegend, USA). They were centrifuged for 5 minutes at 350g at room temperature (RT). The supernatant containing the lysed red blood cells was discarded. The white blood cell pellet was re-suspended in 1ml of freezing medium (RPMI, ThermoFisher, UK, with 10% dimethyl sulfoxide, DMSO, Sigma Aldrich, UK) and stored at –80°C until flow cytometry analysis (Section 2.7).

5.4.5 Toluidine blue staining for murine mast cell localization

Uteri and peritoneal tissue samples from mice were fixed in Carnoy's for 6 hours before placing them in 70% alcohol and embedding them in paraffin (this was performed by the "Shared University Research Facility", SURF Histology team, University of Edinburgh). Paraffin blocks were sectioned using standard methods and 5µm sections were stained with 0.1% toluidine blue (Scientific Laboratory Supplies, East Riding, UK) in 0.5N HCl (Sigma Aldrich, UK) overnight and counterstained with water based-eosin for 2 seconds (ThermoFisher Scientific, UK). Slides were mounted with Permaflour (ThermoFisher Scientific, UK) and sealed with coverslips (Leica Biosystems, UK). Sections were visualized and scanned with the Zeiss Axioscan Z1 (Zeiss, UK). Image analysis was carried out using Zen Blue software (Zeiss, UK).

5.4.6 Flow cytometry and fluorescence-activated cell sorting (FACS)

5.4.6.1 Sample preparation

Following isolation of mouse peritoneal lavages (Section 5.4.4), samples stored at -80°C were thawed at 37°C in a water bath, transferred into polystyrene flow cytometry tubes (BD Falcon, Oxford, UK) and washed with 3ml of filtered FACS buffer (phosphate-buffered saline without calcium and magnesium, PBS,

ThermoFisher Scientific, Paisley, UK; 2% BSA, Sigma Aldrich, Dorset, UK). Samples were centrifuged for 5 minutes at 1000g at 4°C and the resultant cell pellets were re-suspended in 1ml of FACS buffer and filtered to remove cell clumps using filter polystyrene FACS tubes (BD Falcon, UK). The single cell suspensions were counted and viability assessed using the Countess II.

5.4.7 Conjugated antibody staining

Samples were stained in 100µl of FACS buffer with the panel of anti-mouse antibodies; the target proteins, concentrations and their fluorochromes are detailed in Table 2-8. Cell suspensions and antibodies were incubated in the dark, for 30 minutes. Following incubation, 3 ml of FACS buffer were added to the mix and centrifuged for 5 minutes at 1000g at 4°C. See Table 2.9 in materials and methods for antibodies information.

5.4.8 Viability staining

Quantification of dapi was used to assess cell viability before running the sample through the flow cytometer or sorter.

5.4.9 Equipment used for flow cytometry and FACS

Flow cytometry was achieved using a 6 laser BD Fortessa flow cytometer (BD, UK). FACS was performed by the Centre for Inflammation Research Flow Cytometry facility team, using the BD Aria Fusion sorter (BD, UK).

5.4.10 Flow cytometry data analysis

Data gathered from flow cytometry and FACS were analysed using the software FlowJo 8.7 (Flowjo, LLC, USA). Statistical analysis was conducted using GrapPad Prism 6.0. One-way ANOVA was further used for multiple group comparison, followed by Kruskal-Wallis as a secondary test with Dunn's multiple comparisons test. Criterions for significance was $p<0.05$.

5.5 Results

5.5.1 Differentiation of human MCs from blood precursors

During the peripheral blood $CD34^+$ differentiation protocol, a time dependent pattern of proliferation was recorded, with a rapid proliferation during the first 10 days of cell culture.

MC differentiation was monitored on a weekly basis by performing the MC specific metachromatic staining, toluidine blue. Positive cells (purple staining) were first detected after 15 days of culture (end of week 2) as illustrated in Figure 5-5. As the duration of the culture increased, positive cells started to increase in size (21 days – end of week 3) and by the end of the culture they had differentiated into mature MCs, with their distinctive mononuclear morphology and well-organized granules.

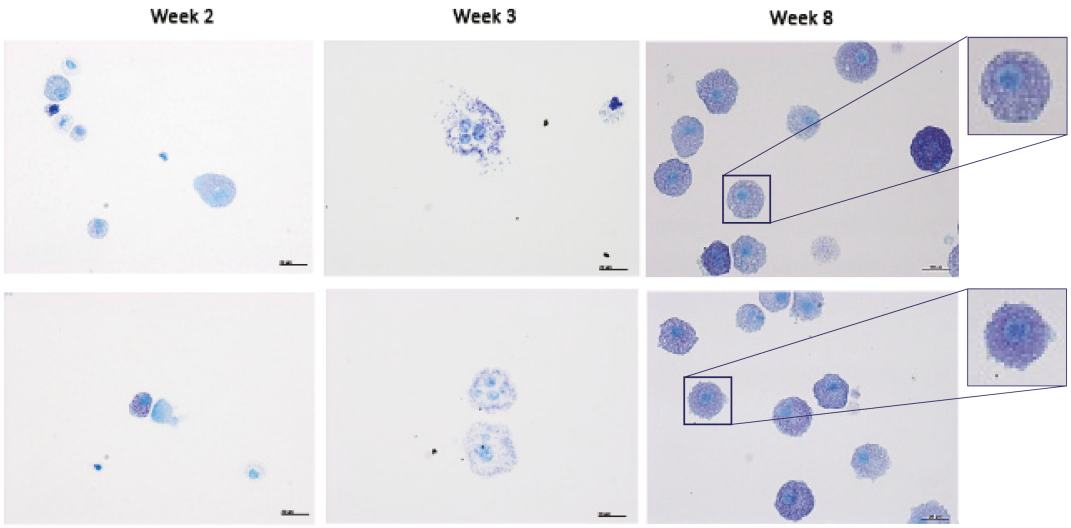

Figure 5-5 Transition of immature mast cells to mature mast cells during* in vitro *differentiation protocol.
Peripheral blood $CD34^+$ progenitor cells were isolated and enrolled into the differentiation protocol described by Kirshenbaum et al (2006). Cationic dye staining was performed throughout the *in vitro* protocol; here are illustrated week 2-3 as intermediate stages and week 8 as the last week of differentiation. MCs started to acquire positivity for toluidine on week 2, but mature MCs were detected only in week 8 samples, which had cells with rounded nuclei and equally distributed purple staining in their cytoplasm, indicating they had developed and well-structured glycosaminoglycan granules. n=3 each stage of differentiation. Scale bar 20μm.

Maturation of MCs was further confirmed by analysing mRNA concentrations of inflammatory markers such as prostaglandin E receptor 2 (*PTGER2*) and glucocorticoid receptor (*GR*), together with the MC specific proteases, tryptase and

chymase encoded by *TPSAB1* and *CMA1* respectively, and the receptors protease-activated receptor 2 (*PAR-2*) and histamine receptor 1 (*HR1*) (Figure 5-6).

Concentrations of messenger RNAs encoded by *TPSAB1* (gene for tryptase α and β isoforms) were significantly upregulated at the end of week 4 and remained elevated at week 8 of culture compared to the undifferentiated cells (week 1). Similarly, *CMA1* mRNA concentrations were significantly upregulated at week 4 and remained elevated at week 8. The MC mediators such as tryptase and histamine mediate their effect through specific receptors, PAR-2 and HR1 respectively. *PAR-2* and *HR-1* mRNA concentrations appeared to remain constant at both week 1 and week 4, however they were significantly upregulated in the cell homogenate from week 8 samples (Figure 5-6-C/D). Together with mRNA profile consistent with MC maturation cells at week 8 of culture also contained *PTGER2* and had significantly higher *GR* mRNA concentrations (Figure 5-6-E/F).

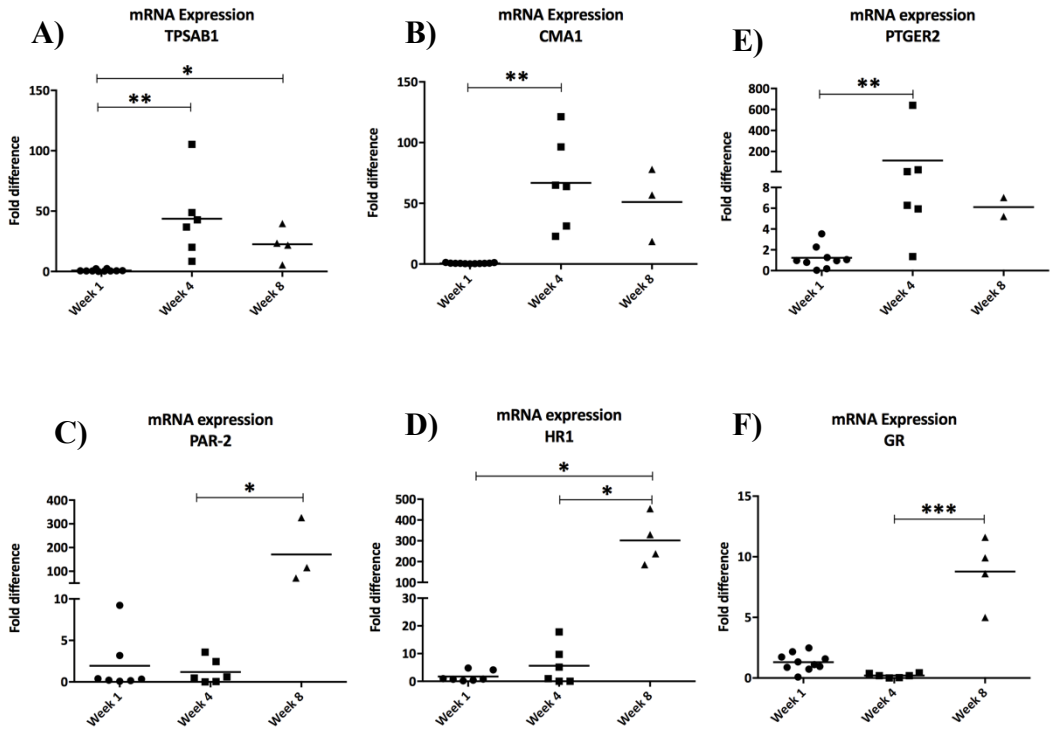

Figure 5-6 Expression of mRNAs encoding mast cell specific proteases and other ininflammatory markers is upregulated in mature peripheral blood derived MCs.
Real-time PCR on different stages of the differentiation of $CD34^+$ into mature peripheral blood derived MCs, identified as significant increased expression of mast cell specific proteases and other inflammatory markers compared to undifferentiated cells. Data expressed as median, One-way ANOVA and Kruskal-Wallis as secondary test with Dunn's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001. Week 1 n=7-10, week 4 n=6, week 8 n=2-4.

These findings are consistent with a "switching on" of an inflammatory expression profile as the MCs differentiated from their precursors, which started to appear after week 4 and increased by week 8, consistent with successful differentiation into mature MCs.

Additional confirmation of MC maturity at the end of culture of a single sample, was achieved by using Western blotting analysis with both isoforms α and β of tryptase detectable in a protein homogenate harvested at week 8 (Figure 5-7).

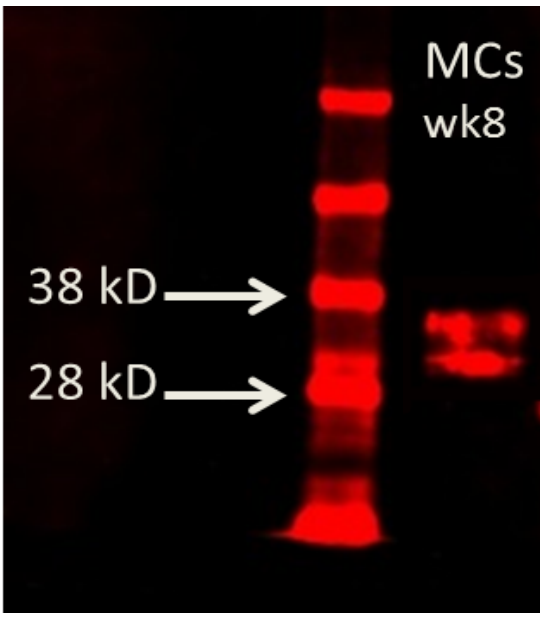

Figure 5-7 Tryptase protein isoforms were detected in the cytoplasm of fully differentiated peripheral blood mast cells. Western blotting analysis of a cell homogenate indicated the presence of the two α and β isoforms of tryptase in the mature peripheral blood derived MCs. n=1

Previous studies have often reported that human MCs express oestrogen receptors (Jensen et al., 2010), and studies described in Chapter 3 highlighted the presence of $ER\alpha^{neg}$ and $ER\beta^{pos}$ MCs in the endometrium. In this study peripheral blood derived MCs differentiated *in vitro* had higher mRNAs concentrations of both *ERα* and *ERβ* in cells at week 4 compared to week 1. Notably, whilst week4 had significantly higher concentrations of *ERα*, these were not detectable in the week 8 samples. In contrast, week 8 cells (mature MCs) had $ER\beta^{pos}$ and $ER\alpha^{neg}$ phenotype (Figure 5-8).

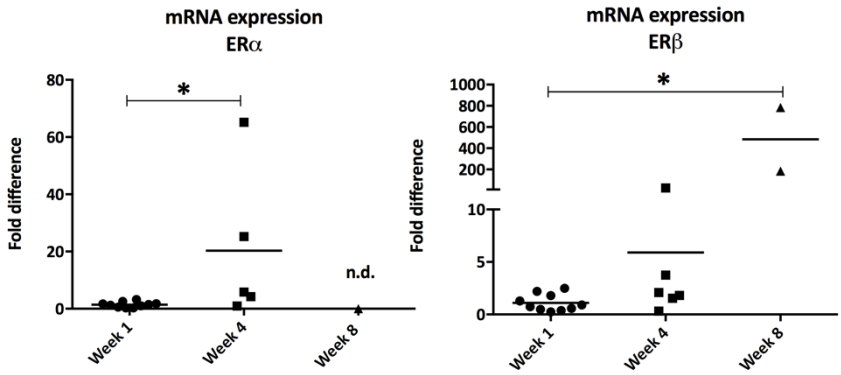

Figure 5-8 Concentrations of mRNA encoding ERα and ERβ in peripheral blood derived mast cells. peripheral blood derived MCs MCs showed an increase of mRNA encoding both ERα and ERβ at week 4 of the differentiation protocol, which was significant for ERα. ERα mRNA concentrations were not detectable in mature peripheral blood derived MCs MCs (week 8). In contrast, significantly higher concentrations for ERβ were detected in mature MCs at week 8. n.d.: not detectable, Data expressed as median, One-way ANOVA and Kruskal-Wallis as secondary test with Dunn's multiple comparisons test. *$p<0.05$, week 1 n=10, week 4 n=6 and week 8 n=2.

5.5.2 Studies using the human mast cell line (HMC-1) as a model system, profiling the phenotype.

The HMC-1 human cell line is widely used as an *in vitro* model for exploring MC behaviour. In the current study, the molecular/cellular phenotype of unstimulated HMC-1 cells was investigated to determine whether it mirrored the profile of inflammatory markers and oestrogen receptors.

When mRNA concentrations in HMC-1 cells were compared to those of the differentiated peripheral blood derived MCs (week 8 of culture), concentrations of all mRNAs were significantly lower (Figure 5-9). This finding would appear to be consistent with the immature/undifferentiated state of HMC-1 cells reported by Nilsson et al. (1994a).

Expression of GR, chymase and tryptase proteins was also investigated (Figure 5-10). Immunocytofluorescence on HMC-1 detected glucorticoid receptor protein in the cell nuclei. Chymase was detected at low intensity in the cell cytoplasm and, HMC-1 cells in culture were immunonegative for tryptase.

Interestingly, tryptase was readily detectable in the HMC-1 culture medium, and not inside the cytoplasm. This finding is in agreement with what was previously shown by Schwartz et al. (2003), where the continuous release of granule content by HMC-1 was monitored.

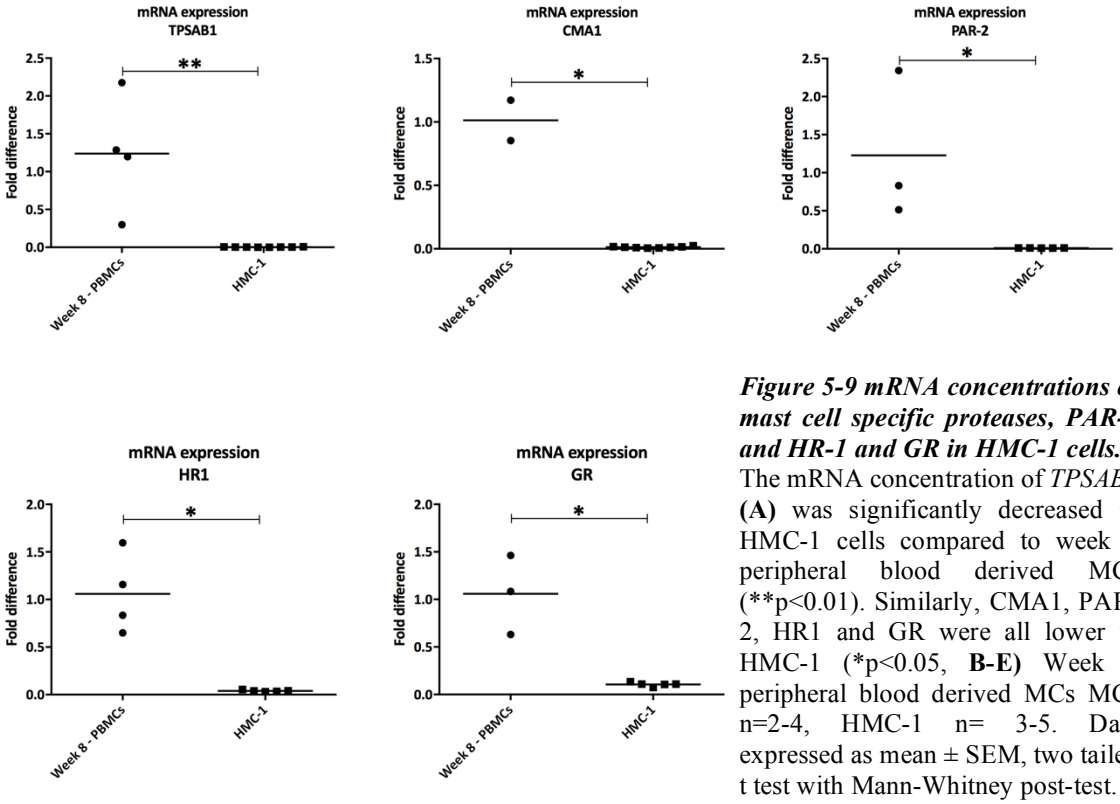

Figure 5-9 mRNA concentrations of mast cell specific proteases, PAR-2 and HR-1 and GR in HMC-1 cells. The mRNA concentration of *TPSAB1* **(A)** was significantly decreased in HMC-1 cells compared to week 8 peripheral blood derived MCs (**p<0.01). Similarly, CMA1, PAR-2, HR1 and GR were all lower in HMC-1 (*p<0.05, **B-E)** Week 8 peripheral blood derived MCs MCs n=2-4, HMC-1 n= 3-5. Data expressed as mean ± SEM, two tailed t test with Mann-Whitney post-test.

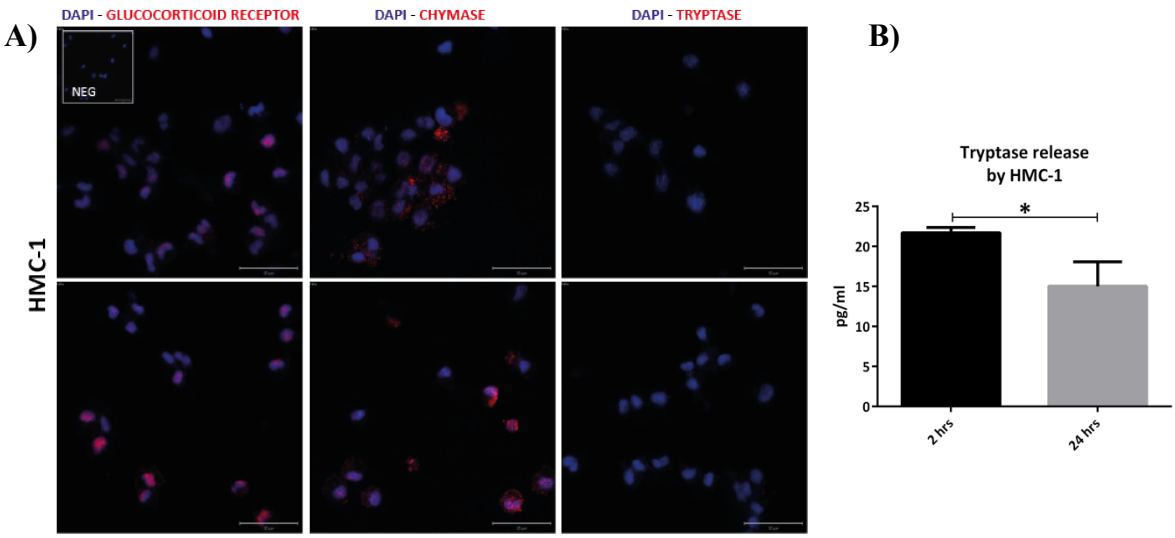

Figure 5-10 Mast cell proteases and glucorticoid receptor expression in HMC-1 and detection of tryptase in culture medium.
A) Immunolocalisation studies demonstrated that HMC-1 express glucorticoid receptor (GR) in their nuclei; low intensity staining for chymase was detected in HMC-1 cytoplasm. Interestingly, tryptase was not detectable in HMC-1 cytoplasm n=5. **B)** Medium from HMC-1 contained detectable tryptase continuously, after 2 or 24 hours of culture. Data expressed as mean ± SEM, two tailed t test with Mann-Whitney post-test, n=5.

HMC-1 cells were also tested for the expression of oestrogen receptors. Real-time PCR detected the expression of mRNAs encoded by *ESR2* the gene encoding (*ERβ)*, while mRNA concentrations for *ERα* (*ERS1*) were undetectable (Figure 5-11-A). This finding was further confirmed by running the RT-PCR products on an 8% (w/v) polyacrylamide gel, with results illustrated in Figure 5-11-B.

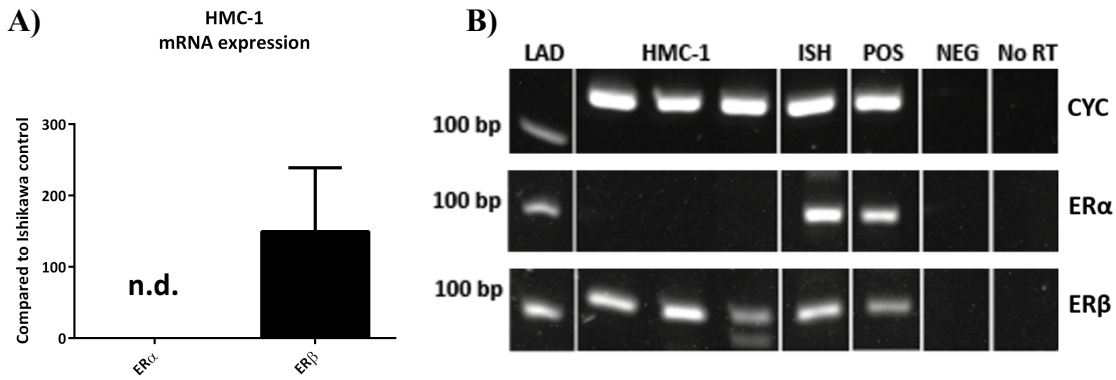

Figure 5-11 Real-time PCR analysis on mRNA expression of ERα and ERβ in HMC-1 cells.
To complement data obtained by real-time PCR **(A)**, the reaction products were run on an 8% acrylamide gel **(B)**. This confirmed that HMC-1 cells did not contain detectable mRNA encoded by *ESR1*, products for ERβ were detectable. n=3, LAD: 100bp Ladder, ISH: Ishikawa cells, POS: positive pooled RNA, NEG: no RNA, No RT: no reverse transcriptase enzyme. HMC-1 for RT-PCR n=5, HMC-1 for gel PCR n=3.

Protein localisation analysis by immunocytofluorescence was carried out using cells fixed on slides following cytospin (Figure 5-12). This technique resulted in detection of ERα and ERβ in the cytoplasm of the positive control Ishikawa cells. Nuclear localisation of ERs requires active shuttling and cytoplasmic localisation would be consistent with cell hypoxia during the cytospin. In the HMC-1 cells no ERα was detected and ERβ was present in the cytoplasm, consistent with the data of Western blots (Figure 5-13).

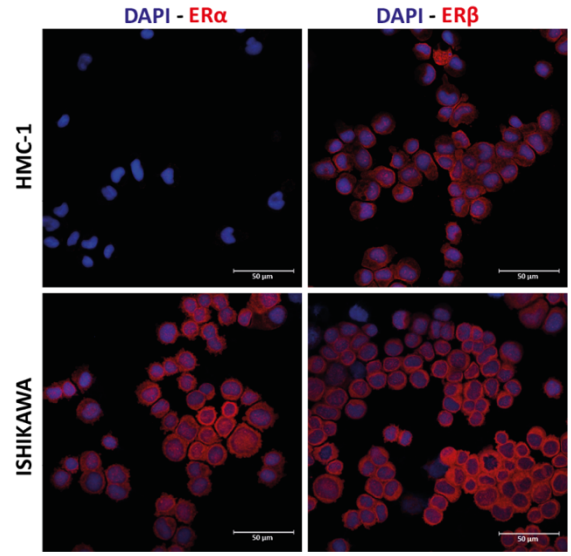

Figure 5-12 Identification of ERα and ERβ proteins on cytospins of HMC-1 cells. Immunocytofluorescence staining showed that HMC-1 cells highly express ERβ (red) and are immunonegative for ERα. Ishikawa cells were used as a positive control for the expression of oestrogen receptors. HMC-1 and Ishikawa n=6.

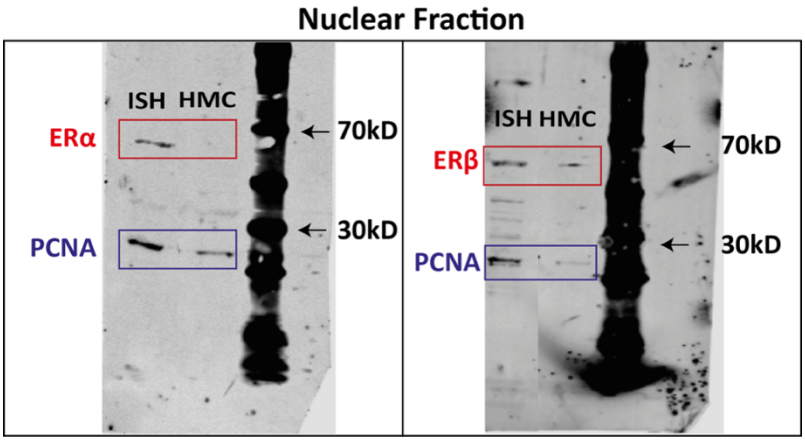

Figure 5-13 Protein quantification of isoform of oestrogen receptor α and β in nuclear fraction of HMC-1. Western blot analysis confirmed that HMC-1 cells do not express ERα but express only the isoform β at protein level as well as mRNA, as shown in Figure 5-11**.** Ishikawa cells were for both ERs as expected. n=1.

5.5.3 Effects of oestradiol on HMC-1 cell culture.

Jensen et al. (2010) reported that oestrogen treatment induced an upregulation in the expression of chemokine receptors in HMC-1. The current study was designed to elucidate the effects of 17β-oestradiol on mRNA expression of mast cell specific proteases and their extracellular release.

Two different doses of 17β-oestradiol (E_2) were assayed, 10^{-7}M and 10^{-8}M, which were chosen to reflect the physiological E_2 concentrations found in the human body. HMC-1 cells were harvested after 2 hours and 24 hours of treatment and in some cultures its pure anti-oestrogen ICI (fulvestrant) was included to determine specificity.

Concentrations of messenger RNA encoded by *TPSAB1* and *CMA1* remained unchanged between the different treatments (Figure 5-14), regardless of the addition of E_2 or fulvestrant. Concentrations of mRNAs were highly variable after 24h; notably in these culture cell number and viability was decreased (data not shown). This may be consistent with a dependency of HMC-1 on serum constituted medium, as the cells were cultured in serum-free medium for 16 hours prior to performing the treatments.

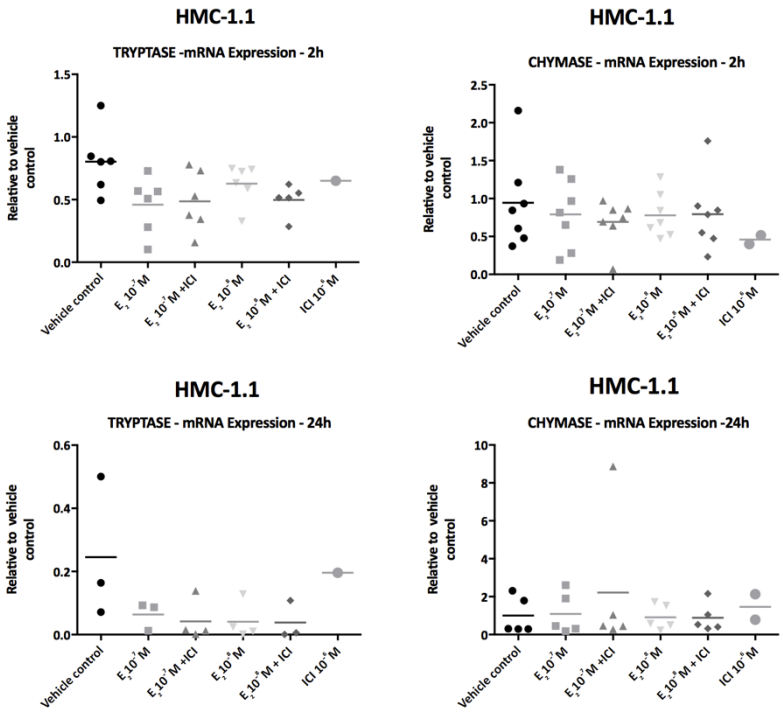

Figure 5-14 mRNA concentrations of genes encoding mast cell proteases in HMC-1 in response to oestradiol remained unchanged.
The mRNA expression of tryptase and chymase, *TPSAB1* and *CMA1* respectively, was investigated after 2 and 24 hours of E_2 treatments. Concentrations of mRNA did not vary with the different combinations of treatments at 2-hour time point. High variability of expression was detected after 24 hours of culture. .2h n=7 (ICI n=2), 24h n=5 (ICI n=1-2). Data expressed as median.

The activation/degranulation profile of HMC-1 cells in response to an E_2 stimulus was also explored. Consistent with results presented above (see Figure 5-10) tryptase release was detected in the conditioned medium of controls, and notably these remained unchanged regardless of treatment (Figure 5-15-A). In the 24h cultures there was an apparent increase in tryptase following addition of E_2 with or without ICI (fulvestrant), but this did not reach significance.

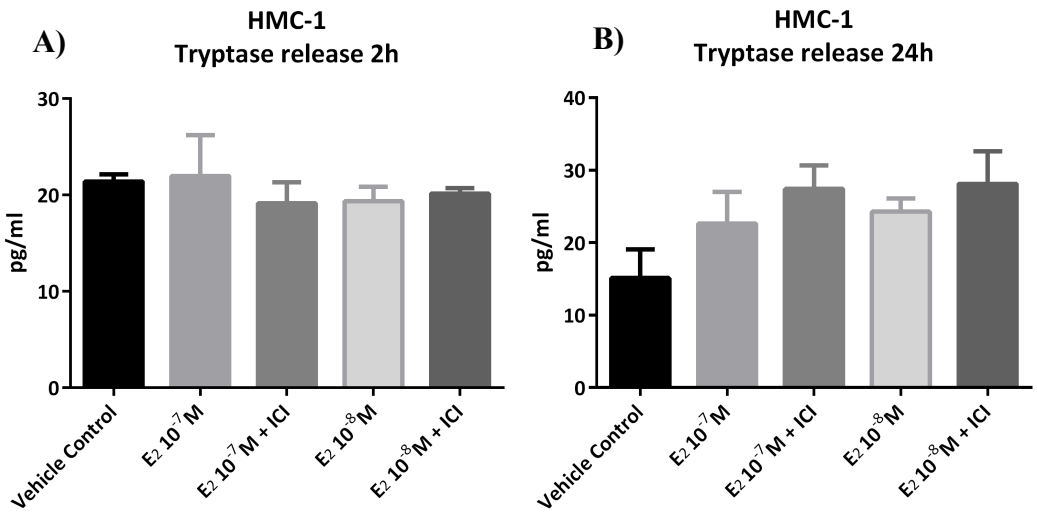

Figure 5-15 Detection of tryptase in HMC-1 culture media after 2 and 24 hours of E_2 treatment. Tryptase levels were readily detected after 2 hours of culture and remained unchanged within the different groups, as shown in panel (**A**). After 24 hours of E_2 and ICI (fulvestrant) treatments, levels of tryptase appeared to have slightly increased but this was not significant or was blocked by ICI (**B**). 2h n=2-4, 24h n=4.

5.5.4 Localisation of mast cells in the uterus of murine models of menstruation.

Mast cells have been documented to be part of the immune cell population of the uterus, both in the human myometrial and endometrial compartments, as shown in Chapter 3. A few studies have investigated uterine MCs during the oestrus cycle in the mouse, which reported a peak in MC numbers during a specific phase of the cycle (oestrus), and during the first weeks of implantation (Woidacki et al., 2013b).

To complement and extend studies on human uterus reported in Chapter 3 of this thesis, methods were developed to localise MCs in the mouse uterus during the oestrus cycle. This was a necessary first step, as antibodies against human tryptase and chymase do not cross react with mouse proteins. Acidic toluidine blue staining using Carnoy's fixed uterine horns revealed the spatial and temporal pattern of localisation of MCs in the mouse uterus. MCs were most abundant in the inner myometrial compartment (towards the uterine cavity); no MCs were detected in either the stromal or the epithelial compartments of the endometrium. Uterine MCs were not abundant and their numbers only varied slightly during the oestrus cycle, showing an apparent

increase in oestrus and early metestrus and a reduction at diestrus; results are illustrated in Figure 5-16. MCs were also localised next to uterine vessels.

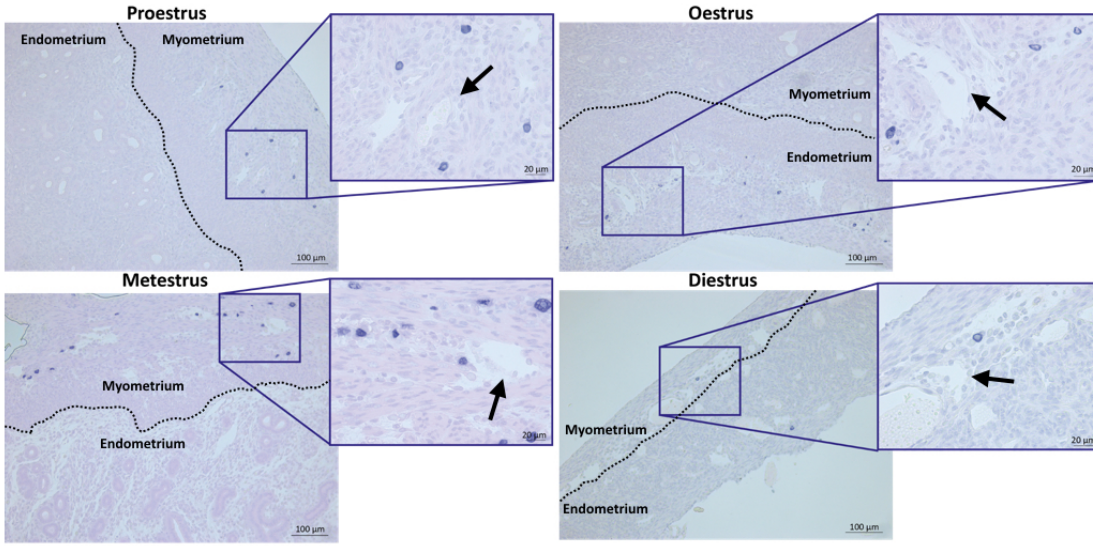

Figure 5-16 Mast cells detected in the murine uterus during the different phases of oestrus cycle. Toluidine blue staining performed on Carnoy's fixed sections revealed the spatial and temporal localization of murine uterine MCs across the oestrus cycle. MCs were resident in the myometrial compartment, near the blood vessels (black arrows). MCs were not detected in the endometrium at any stage of the cycle. An increase in number of MCs was apparent at oestrus and early metestrus phases. Proestrus n=4, oestrus n=5, metestrus n=3 and diestrus n=3.

The location and number of MCs was also investigated for the first time in a mouse model of menstruation. Uterine horns were collected at two different time points, 24 and 48 hours after progesterone withdrawal to model both tissue breakdown and repair as MCs have been predicted to be involved in both these processes in the human endometrium.

Unexpectedly, toluidine blue positive MCs were not detected the endometrial compartment at either time points studied (Figure 5-17). In these tissue sections MCs remained in close proximity to blood vessels in the myometrium, consistent with their localisation in the oestrus cycle sample.

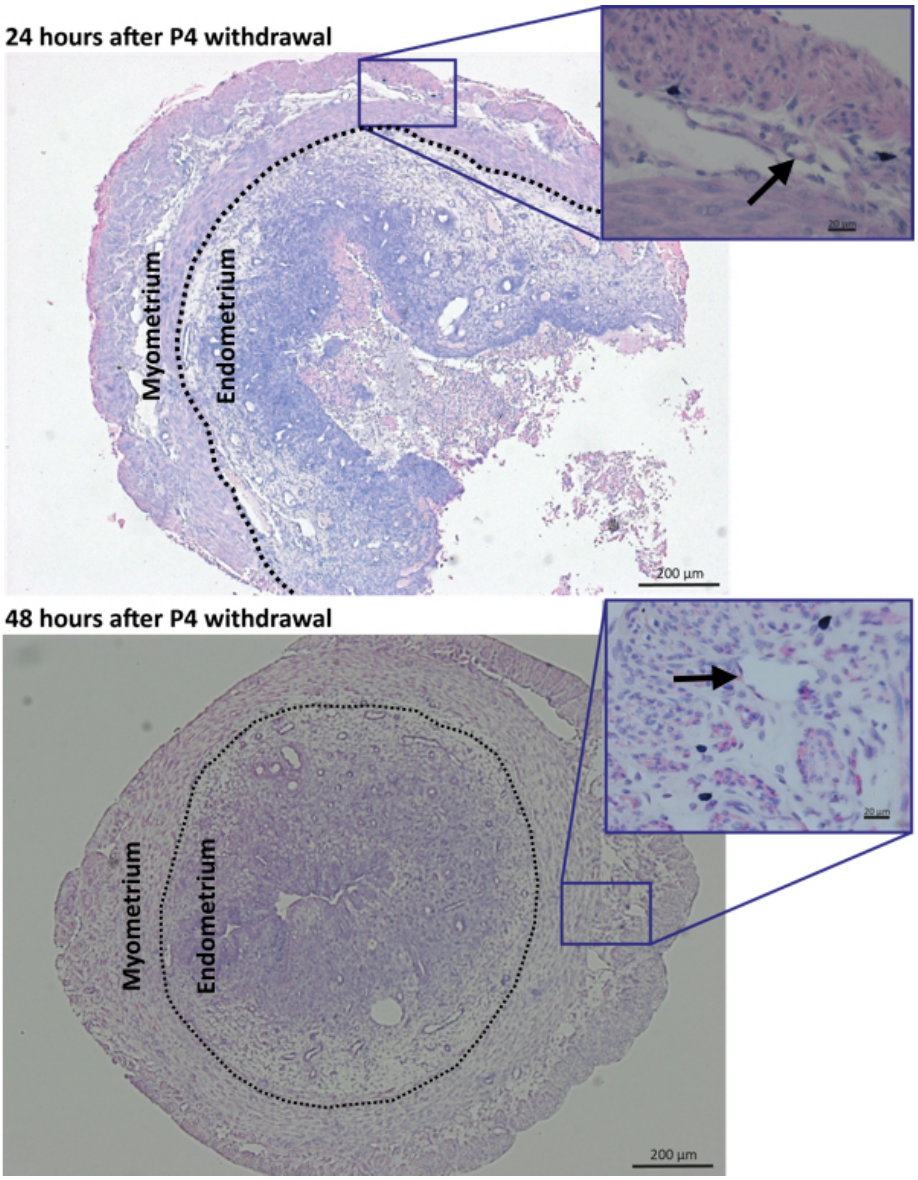

Figure 5-17 Localisation of mast cells in uterine cross sections 24 and 48 hours after progesterone withdrawal.
Cationic staining with toluidine blue revealed that MCs are located in the peripheral layer of the myometrium; but were not detectable in the endometrium during tissue breakdown and remodelling. Black arrows indicate blood vessels. T-24h n=10, T-48h n=2

5.5.5 Investigation of mast cells in a mouse model of endometriosis

5.5.5.1 Mast cells in endometriosis-like lesions

There is evidence for the presence of MCs in peritoneal lesions of patients suffering from endometriosis (Sugamata et al., 2005), which was extended during studies in Chapter 4. The presence of MCs in endometrial-like lesions from rodent models of endometriosis has however never been investigated.

The current project explored the presence of MCs using our in-house murine model of endometriosis (Greaves et al., 2014a). Strikingly, MCs were readily detected in the endometrial-like lesions harvested at the end of the protocol, a representative image is presented in Figure 5-18. Typically, the lesions recovered at the end of the study, look heterogeneous. They are often constituted by endometrial stroma, fat and fibrotic tissue and a peritoneal fraction, as reported by Greaves et al., 2014. During the current study, MCs were predominantly located in the stromal compartment, which is probably of endometrial origin, and in the fibrotic and fat sites, as shown in Figure 5-19.

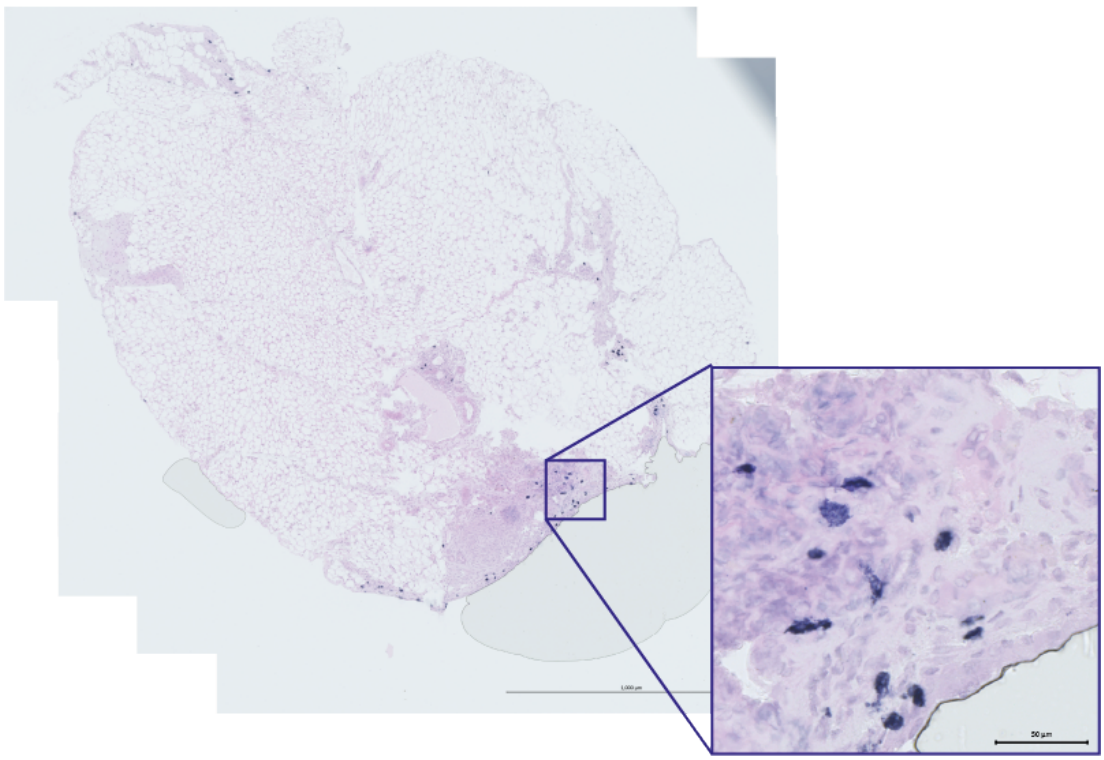

Figure 5-18 Representative toluidine blue staining on endometrotic lesion in the murine model of endometriosis
Mast cells were easily detected in the "endometrial-like" lesions, as shown by the purple staining. MCs were often located in stromal-like fraction and in close proximity with fat cells. Lesions screened n=48 Scale bars: 200μm in **(A)** and 50μm in **(B).**

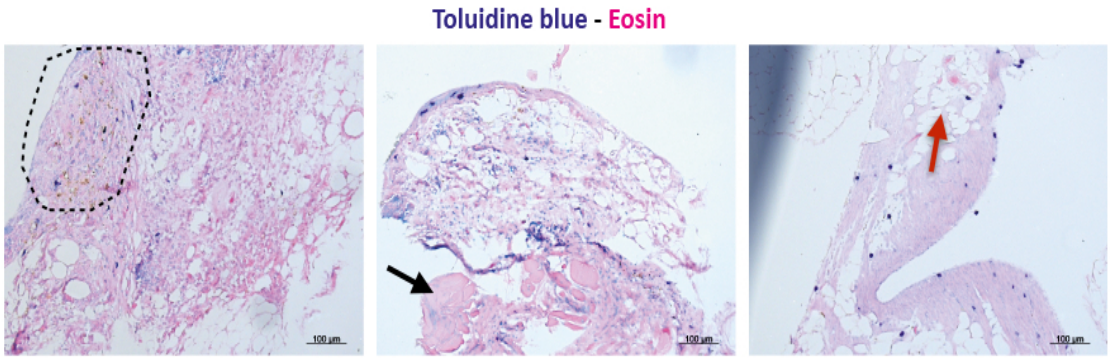

Figure 5-19 Localization of mast cells in endometriotic lesions in a mouse model of endometriosis. Acidic toluidine blue staining enabled the detection of MCs in the "endometrial-like" lesions harvested at the end of Greaves et al. (2014) protocol. Purple cells were easily observed throughout the tissue sections, with the specific localization in the stromal endometrial compartment. MCs were often found in fibrotic and fat tissue (black dotted line surrounds brown colour for haemorrhagic staining, red arrow indicates fat tissue). Peritoneal tissue fraction indicated by black arrow within the lesion. Lesions n=48. Scale bar 100µm.

5.5.5.2 Mast cell localisation in peritoneal fluid in a mouse model of endometriosis

To complement the study conducted on MCs in women affected by chronic pelvic pain and endometriosis detailed in Chapter 4, MCs were also examined in the peritoneal lavages of mice undergoing the endometriosis model.

Firstly, the presence of MC was confirmed within the immune cell populations present in the peritoneum using toluidine blue staining of cytospins of prepared from peritoneal lavages. Figure 5-20 illustrates MCs identified in peritoneal lavage and shows that they were representative of two murine subtypes: connective and mucosal. This classification is based on the different colour reaction after toluidine blue staining, "connective" type cells acquiring a more intense purple colour compared to the

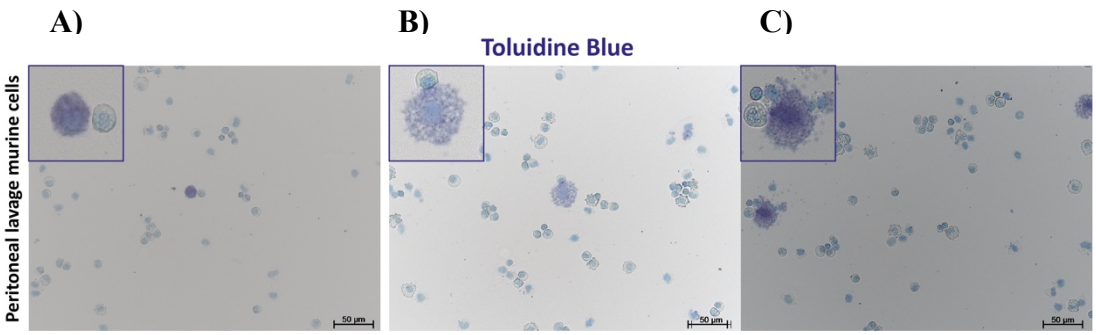

Figure 5-20 Identification of mast cells in peritoneal lavages using toluidine blue staining. Toluidine blue staining of peritoneal cell cytospins showed the presence of both mucosal and connective type murine MCs, which differ on the intensity of staining. Example of co**(A,C)** and mucosal type in **(B)**. Scale bar 50µm, n=3.

"mucosal" type. The distinctive colouration detected is due to the different composition of their granules, as previously reviewed by Galli et al. (2011).

This study was also able to quantify the number of MCs present in the lavage of different groups of mice in the endometriosis model. MCs usually represent a very small percentage of $CD45^+$ cells; therefore, an effective exclusion gating strategy is required to perform accurate quantification. Figure 5-21 shows a representative gating strategy, including two plots with the FMO gates (fluorescence minus one), in this case, showing the specific surface makers for MCs: c-kit and FcεRIα. The use of FMO

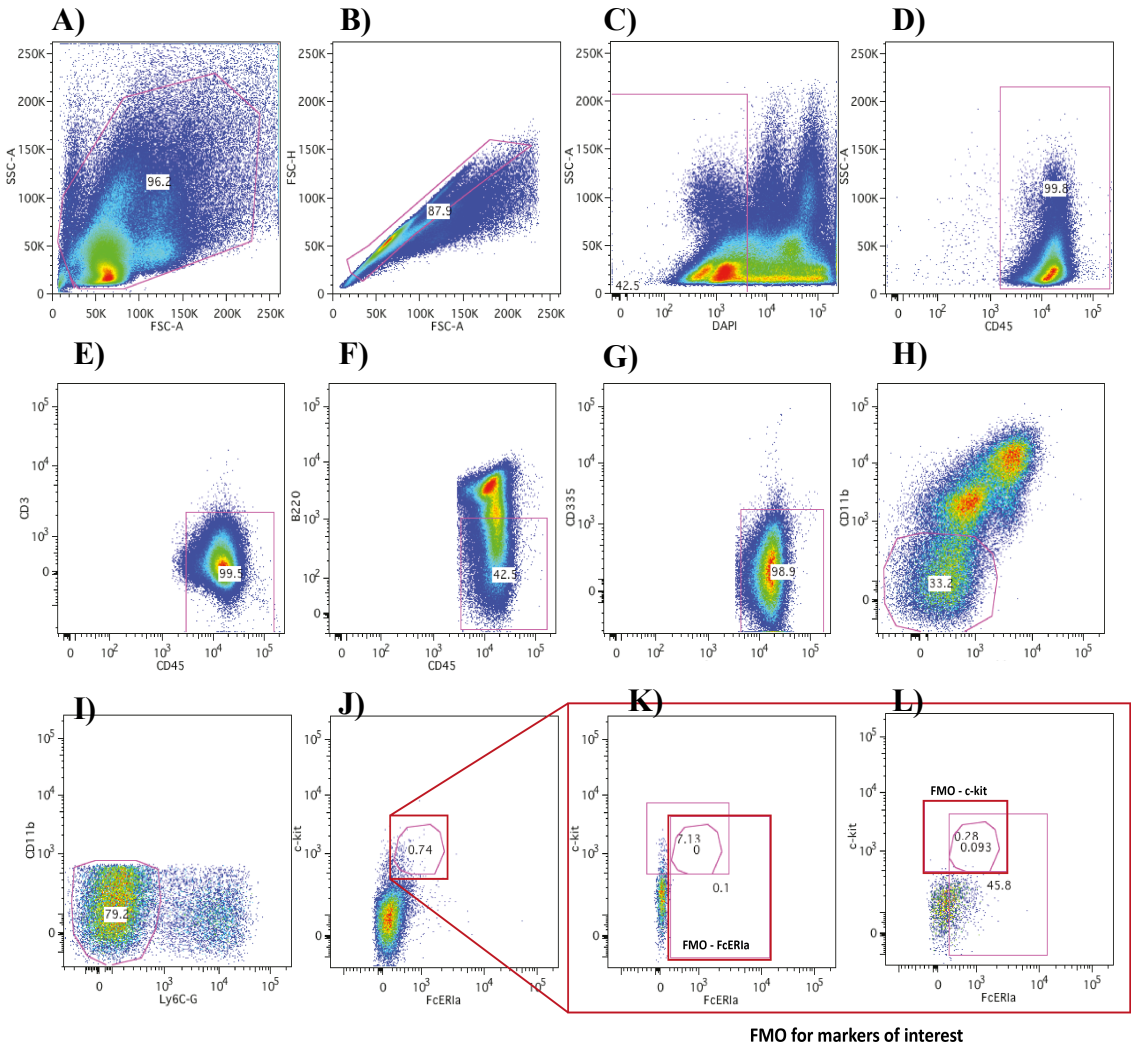

Figure 5-21 Representative gating strategy for quantifying MCs in peritoneal lavages.
An exclusion criterion gating strategy was employed during flow cytometry studies. After size and doublets were excluded **(A-B)**, cells were gated on dapi negative **(C)**, $CD45^+$ **(D),** $CD45^+/CD3^-$ **(E,** T cells excluded), $CD45^+/B220^-$ (**F**, B cells), $CD45^+/CD335^-$ (**G**, NK cells). For excluding macrophages, cells were gated on CD11b-F4/80 negative **(H)** and Ly6C-G **(I)**. Finally, mast cells were identified using the unique combination of c-kit (CD117) and FcεRIα antibodies, shown in panel **J**. Panels **K** panels show the FMOs used. These gates were applied on all the analysed samples

was extremely important for the evaluation of the small number of MCs in the peritoneal lavages, avoiding any artefact during the gating. The markers used to fulfil the "exclusion" criteria were: CD3 for T cells, B220 for B cells, CD335 for natural killers and CD11b-F4/80-Ly6C/G for macrophages, Ly6G excluded also neutrophils.

This method and gating strategy allowed the frequency of MCs as a percentage of the total live $CD45^+$ cells (blood cells) to be identified. MCs represented on average <0.3% of total live $CD45^+$, and this percentage was lower in animals treated with E_2 alone, animals treated with PBS as a control groups, and the animals in which endometriosis was simulated when compared to intact animals (Figure 5-22-A). Mice injected with intraperitoneal PBS showed a significantly lower percentage of MCs, if compared to any of the other groups. This finding was similar to the one that was firstly reported by Katakura et al. (1988), where ip injection of distilled water induced a complete depletion of MCs in the peritoneal cavity. Peritoneal MCs, therefore, appear to be sensitive to low osmotic pressure, following the addition of only solution and not "particles" (cells) in the environment. This event is not caused by endometrial tissue injection in endometriosis animals, because endometrial cells together with PBS suspension are introduced into the peritoneum, without changing the total osmotic pressure.

Quantitative measurement of MC proteases was also performed on the peritoneal lavage solutions (Figure 5-22-B). Concentrations of tryptase and chymase were lower in the lavages from treated animals compared to naïve animals, however these did not reach statistical significance due to the large variation in controls.

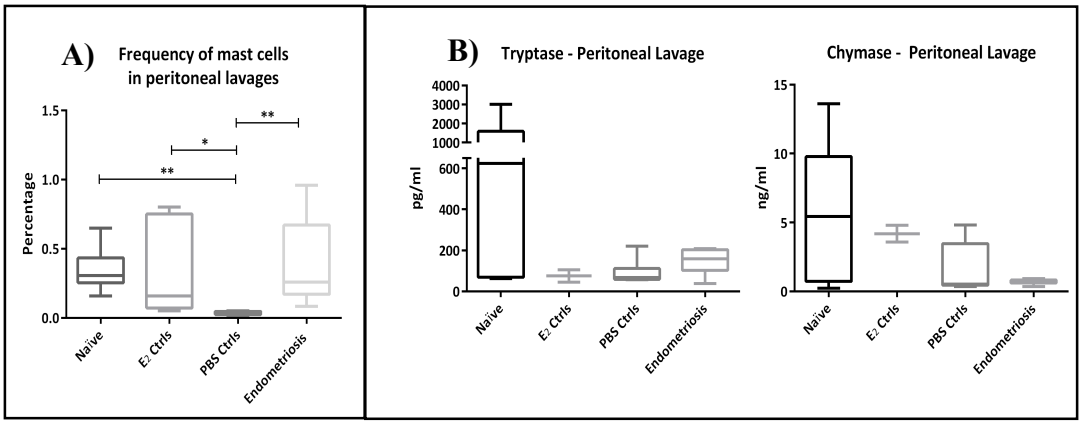

Figure 5-22 Frequency of mast cells and concentrations of proteases in the peritoneal lavages.
A) MCs were detectable and quantifiable in peritoneal lavages from different groups of animals. E_2 and endometriosis groups showed lower median compared to näive controls. Significantly decreased was the percentage of MCs in lavages from PBS controls. One way ANOVA and Kruskal-Wallis as secondary test with Dunn's multiple comparisons test. *p<0.05, **p<0.01, naïve n=10, E_2 ctrls n=9, PBS ctrls n=6, Endometriosis animals n=15. **B)** Levels of tryptase and chymase were measured in the peritoneal lavages. Lower concentrations of both proteases were detected in E_2, PBS and endometriosis groups. Naïve n=6, E_2 ctrls n=2, PBS ctrls n=7, Endometriosis animals n=9.

5.6 Discussion

Female sex steroid hormones have often been suspected to exert a stimulatory effect on tissue resident MCs. This assumption seems plausible due to the clear difference in incidence and severity of MC related disorders between women and men (Bonds and Midoro-Horiuti, 2013). There are reports that both human and murine MCs express oestrogen receptor isoforms (Zhao et al., 2001, Zaitsu et al., 2007, Jensen et al., 2010). Additionally, the studies in this thesis show that human uterine MCs are $ER\beta^{pos}$ and $ER\alpha^{neg}$.

The current study set out to evaluate the methods currently available to investigate the effects of oestrogen on human MCs using *in vitro* model systems that included the use of *in vitro* matured peripheral blood-derived MCs and use of the leukaemia cell line HMC-1. Both approaches resulted in novel findings.

Interrogation of oestrogen receptor expression over the time course of peripheral blood derived MCs differentiation revealed dynamic changes in mRNA concentrations for both *ESR1 (ERα*) and *ESR2* (*ERβ*) with both present at the mid-differentiation stage (week 4), but fully mature peripheral blood derived MCs being $ER\beta^{pos}$ and $ER\alpha^{neg}$. To my knowledge, this is the first study that has investigated oestrogen receptor expression in peripheral blood derived MCs. This result was consistent with *in situ* studies reported in Chapter 3, and suggests mature *in vitro* derived peripheral blood derived MCs are a suitable model system.

Notably, it has been shown that oestrogen can stimulate the differentiation of $CD34^{+}$ progenitor cells into megakaryocytes via ERα and ERβ. Similar to our culture system, expression of ERα was suppressed at later stages of the *in vitro* culture, while ERβ increased and remained constant in mature megakaryocytes (Bord et al., 2004). It can be speculated that oestrogens acting via ERα may be involved in the recruitment of progenitors and early MC development, while ERβ may be responsible for exerting oestrogen dependent effects within mature MCs. This $ER\alpha^{neg}$ and $ER\beta^{pos}$ phenotype detected in the peripheral blood derived MCs mimics that of other immune endometrial cells, including natural killer cells (uNKs) and $CD68^{+}$ macrophages (Henderson et al., 2003, Thiruchelvam et al., 2013).

Whilst peripheral blood derived MCs were formed, the differentiation protocol was lengthy and cell numbers were very low. To overcome this limitation the leukaemia-derived HMC-1 cell line as established by Butterfield et al. (1988), was also investigated. It has been reported that HMC-1 cells are able to respond to oestrogenic stimuli via ERα (Zaitsu et al., 2007, Jensen et al., 2010). Several techniques were employed to explore oestrogen receptor expression in HMC-1 cells obtained by Dr Butterfield during the current study. Using multiple tests, including RT-PCR, gel PCR, Western blotting and immunofluorence the HMC-1 cells in our culture work were $ER\alpha^{neg}$ and $ER\beta^{pos}$, which supported the peripheral blood derived MCs data but appeared at odds with previous reports. The HMC-1 did not show significant response to physiological oestradiol concentrations after 2h or 24h of treatment, the rapid non-genomic action that was suggested by Zaitsu et al. (2007).

The presence of MCs in the uterus and their oscillations during the menstrual and oestrus cycle has been described in humans (Drudy et al., 1991a, Jeziorska et al., 1995) and other mammals, firstly in the hamster (Harvey, 1964) and later in the mouse (Padilla et al., 1990). In humans, we and others have shown MCs are most abundant in the basal layer of the endometrium and in the myometrium (Sivridis et al., 2001). Mori et al. (1997a) reported that MCs do not change in number during the different phases of the menstrual cycle, following the fluctuation of the ovarian sex hormones and therefore MCs are considered a tissue resident immune cell population. In the mouse, MC number has been reported to vary during the four phases of the oestrus cycle, reaching a peak during oestrus (Woidacki et al., 2013b). It has been postulated that a higher number of uMCs during oestrus may correspond to the maximum circulating levels of 17β-oestradiol and to the minimum circulating levels of progesterone (Fata et al., 2001).

This was the first study, to my knowledge, that has used toluidine blue staining on Carnoy's fixed murine uterus sections to detect tissue resident MCs. Other fixatives, such as formalin derived (PFA or NBF), did not permit the detection of the full complement of murine MCs. For example, mucosal type MCs are reported to be "formalin sensitive", due to their low content of heparin in the architecture of the granules (Irani and Schwartz, 1989, Sridharan and Shankar, 2012). In this study, similar results to that of Woidacki et al.'s (2013b), which employed PFA fixed tissue, were obtained. Murine MCs reside primarily in the myometrial compartment of the uterus, located around blood vessels and their number and localisation did not appear to change with the phase of the cycle. No MCs were detected in either the stromal or epithelial compartments of the endometrium. MC number was higher in oestrus tissues. For the first time, due to the more appropriate fixation method, a notable increase in early metestrus tissue was observed, suggesting a stage specific influx of mucosal type MCs.

The role of MCs in the menstruation process has been postulated, due to their potential for biosynthesis of metalloproteases (MMPs) (Schwartz, 1994). MCs are known to be part of the leukocyte population of the human endometrium and they are reported to be activated before tissue breakdown (Jeziorska et al., 1995). My previous

data, reported in Chapter 3, has revealed that mature uterine MCs express the isoform β of the oestrogen receptor, supporting evidence that MC activation may be hormone dependent. Moreover, Zhang et al. (1998) demonstrated that MCs can stimulate the local production of MMPs by primary endometrial stromal cells, suggesting MC involvement during the tissue breakdown process.

Chapter 5 study was set up to explore the location and number of MCs in a well-established murine model of menstruation (Cousins et al., 2014). The model elegantly mimics the human menstrual cycle with cellular proliferation during the early stage of the protocol in response to oestrogen injections, angiogenesis and cellular differentiation in response to the progesterone implant and induction of decidualisation: and finally tissue breakdown after progesterone withdrawal. Most importantly, this model has been used to explore dynamic changes in other uterine immune cell populations including monocyte, macrophages and neutrophils.

Unexpectedly, in this mouse model MCs were not identified in the shed or the newly proliferating stromal compartment of the endometrium 24 hours after progesterone withdrawal, or at the stage of complete endometrial regeneration (48h time point). Regardless of hormonal manipulation, MCs were detected within the smooth muscle fibres of the myometrium, as in normal cycling animals. This model thus does not shed light on whether MCs are involved in endometrial shedding and remodelling during menstruation in women. On the contrary, due to their constant and unchanged localisation in the myometrium, MCs may be associated with regulation of muscular contractions, which the uterus spontaneously undergoes, both in humans and in rodents (Talo and Karki, 1976, Bulletti et al., 2000). Smooth muscle fibres, including myometrial fibres, are a powerful source of stem cell factor (Mori et al., 1997a), which critically regulates the migration of MC precursors and promotes MC proliferation and maturation (Galli et al., 1993). Notably, it has been demonstrated that MC mediators secreted after degranulation alter myometrial fibres, augmenting spontaneous contractions (Bytautiene et al., 2003, Bytautiene et al., 2004). Moreover, in mice oestrus and early metestrus are described as the phases during which more frequent myometrial contractions are reported (Crane and Martin, 1991). Therefore from these data it is worth considering whether MCs may be involved in myometrial

hyperactivity during the perimenstrual phase, reported to occur during painful menstruation (Dawood, 2006).

The results reported in Chapter 4 along with previous literature on MCs have described their infiltration into the stroma of human endometriotic lesions (Matsuzaki et al., 1998b, Sugamata et al., 2005). Prior to this study the presence of MCs in rodent models of endometriosis has not been reported. The current investigation identified MCs in endometrial-like lesions in a well-established mouse model of endometriosis (Greaves et al., 2014a). Not only were toluidine blue positive MCs detectable but they appeared to be present in relatively high numbers in the endometriotic "lesions", detectable in the stroma, as well as the fat and fibrotic tissue that contributed to the microenvironment of the lesions.

This result was rather unexpected, as mature MCs were not detected in the endometrium during the different time points of the menstruation protocol and this is recapitulated with the "donor" tissue used to induce lesions. It is possible that MC progenitors present in the endometrial stroma might have differentiated into mature MCs after the establishment of the lesion. Other scientists have reported that the endometrial stromal compartment contains a fraction of cells that are immunopositive for $CD34^+$ (Lindenmayer and Miettinen, 1995, Mai et al., 2008). Therefore, the donor material injected into recipient animals may have brought a pool of MC progenitors into a different and inflamed environment, which then, with a pro-inflammatory array of molecules, have differentiated into MCs.

Furthermore, in agreement with the patient data reported in Chapter 4, results obtained using the endometriosis model have shown an apparent decrease in the number of MCs and concentrations of tryptase and chymase, in the peritoneal lavages of animals that had been ovariectomised and treated with E_2 and undergone the endometriosis model. This might suggest that a "hyperalgesic state" is present in the abdominal cavity, where lower numbers of cells can trigger higher pain/discomfort. It has been demonstrated that MCs can be critical players in the pathogenesis of chronic and neuropathic pain (Chatterjea and Martinov, 2015). Colleagues (Greaves et al. paper under review) have showed during the aforementioned model that the recipient animals experience significant discomfort and hyperalgesia, compared to naïve

controls. Thereby the reported increase of number of MCs in endometriotic lesions during the current study supports the veracity of the model and the potential involvement of MCs in endometriosis-induced pain.

Limitations have been encountered during the current *in vitro* and *in vivo* studies. Derivation of peripheral blood derived MCs was an extremely complex cell culture protocol which was time consuming and expensive, despite resulting in MCs that showed a similar expression pattern to mature uterine MCs. The HMC-1 cell line generated large numbers of cells instantly however these presented as immature inflammatory state (low tryptase and chymase) which limited their utility as a model for tissue resident MCs. The main obstacle for mouse models in MC research, is the lack of commercially available antibodies that specifically detect MC proteases. Thereby, only toluidine blue dye can be employed for for the identification of murine MCs, which doesn not allow for a deeper investigation of the protein content of tissue resident MCs.

In summary, using these different *in vitro* cell models this study demonstrated that MCs can respond to oestrogen action since both peripheral blood derived MCs and HMC-1 express ERβ, a finding that was in agreement with MCs detected *in situ* during studies reported in Chapter 3. Importantly, these findings do not support previous studies claiming MCs to be $ER\alpha^{pos}$. The preliminary studies in mouse models offer a valid platform for further studies on the implications of MCs in pain associated with endometriosis or menstruation.

Chapter 6 Final discussion

6.1 Introduction

The cellular and extracellular composition of the human endometrium undergoes substantial remodelling during the menstrual cycle. Ovarian derived steroid hormones, oestrogen and progesterone, and their fluctuating serum levels are responsible for the dynamic tissue reorganization. The endometrial immune cell population has been implicated in regulation of tissue adaptation across the different phases of the cycle (Evans and Salamonsen, 2012b) with both the total number of leukocytes and the relative numbers of most immune cell subtypes varying according to the different stages of the menstrual cycle. Endometrial leukocytes include uterine natural killer cells (uNKs), macrophages, eosinophils, neutrophils and mast cells (MCs). While the number of macrophages and natural killer cells increases immediately after ovulation and during the decidualisation process, the influx of eosinophils and neutrophils occurs during the late secretory and pre-menstrual phases (Kamat and Isaacson, 1987, Kammerer et al., 2004). In contrast, the number of mast cells has been reported to be constant regardless of the phase of the menstrual cycle (Salamonsen et al., 2002). Whereas other uterine leukocytes have been extensively studied, only a few studies have investigated the phenotype of the mature mast cells resident in the human uterus.

Endometriosis is common gynaecological disorder that is often associated with debilitating chronic pelvic pain (CPP). It is an oestrogen-dependent inflammatory condition characterized by growth of endometrial tissue in ectopic locations, generally on the peritoneum (Nisolle and Donnez, 1997). Ectopic lesions are reported to develop small diameter nerve fibres typical of afferent sensory innervation (Mechsner et al., 2007), and it has been postulated that these nerve endings are activated by inflammatory mediators within the endometrial fragments, which may contribute to endometriosis-associated pelvic pain (Triolo et al., 2013). MCs are immune cells that have recently been proposed as key cellular sensors in inflammation and immunity. When stimulated, MCs release a wide range of mediators and may orchestrate an aberrant inflammatory response. MC mediators can directly influence tissue responses by resident cells, fibroblasts and endothelial cells, and may interact with other immune cells. New evidence indicates the involvement of MCs in the development of pain

processes as well as in the transition from acute, to chronic and neuropathic pain (Graziottin et al., 2014). The important feature of MC biology is the tissue dependency of their mature phenotype, with MC differentiation from precursors being strongly influenced by the surrounding tissue microenvironment (Metcalfe et al., 1997). A number of lines of evidence have suggested that female sex steroid hormones may influence MC phenotype but this has never been comprehensively investigated in the uterus even though this is a hormone-dependent organ.

During the current study, novel insights into the biology and potential impact of steroids on uterine MCs were gained from detailed studies using tissues from women who were considered to have a normal uterine environment and those attending a chronic pelvic pain clinic, some of whom had endometriosis. To complement these studies both *in vitro* (cell based) models and a mouse model of endometriosis were investigated.

6.2 Uterine mast cells and their tissue specific phenotype

In chapter 3, it was demonstrated that the phenotype and activation profile of MCs resident in the human uterus are influenced by the fluctuation of sex hormones during the different phases of the menstrual cycle. The phenotype of MCs was not only phase-dependent; it appeared different between the three layers of the human uterus. For the first time, the presence of the MC_C subtype in the human uterus was shown. MC_C were only detected in the basal endometrium and in the myometrium specifically during the early, mid secretory and menstrual phases.

During the 1990s studies on the distribution of uterine MCs used a range of metachromatic staining techniques, such as toluidine blue and alcian blue stains (Drudy et al., 1991a, Drudy et al., 1991b). All studies encountered problems during the quantification of positive cells due to difficulties in detecting low granule content after degranulation. Notably, the pioneering investigation on uterine MCs was the one conducted by Jeziorska et al (1995). In this study they used antibodies specific for the MC proteases tryptase and chymase to conduct immunostaining of sections throughout the normal menstrual cycle of fertile women. It was reported after quantification of

tryptase immunopositive cells that whilst MCs were members of the tissue resident population of endometrial leukocytes, they didn't vary in number across the different phases. Staining for tryptase and chymase was conducted using serial tissue sections and the authors reported that uterine MCs belonged to the classical protease-content subtypes: MC_{TC} and MC_T a finding that was later confirmed by Mori et al. (1997b) by using double staining with alkaline phosphatase for chymase and DAB for tryptase. However, neither of these studies reported detection of the MC_C subtype. Therefore, the current study is the first to detect all three subtypes of MCs in the uterine compartments by using the sensitive method of double immunofluorescence coupled with the tyramide amplification system which is reported to have 1000-fold signal amplification compared to DAB detection.

Notably, uterine MCs showed a different phenotype based on their locations in the different compartments of the uterus. As confirmation of previous studies, MC_{TC} were predominantly resident in the basal endometrium and in the myometrium and MC_T were found in functional endometrium. The rare MC_C type was detected in the basal endometrium and myometrium and completely absent in the functional layer. These findings reinforce the principle that tissue specific MC phenotypes can exist within different regions within the same organ. It is well known that the three uterine compartments are different to each other in terms of cellular composition and cytokine/chemokine microenvironments. Interestingly, the majority of MCs were detected around the endometrial-myometrial junction. Previous studies have demonstrated that a large number of $CD34^+$ MC progenitor cells reside in this area of the tissue independent of phase of the menstrual cycle (Cho et al., 2004, Mai et al., 2008). MCs were also in close proximity to smooth muscle fibres, the main source of stem cell factor, and a vital mediator for MC maturation and survival, specific growth factor of this microenvironment (Zhang et al., 1996).

Another important aspect that is influenced by the microenvironment is the activation profile of MCs. Thus, the phenotype and activation/degranulation profile of uterine MCs appeared to be menstrual phase dependent, being most evident specifically during early and mid secretory stages, suggesting angiogenic processes taking place in the endometrium at this time may be mediated by MC degranulation. It is well known that histamine and prostanoids from MCs are important in the regulation of the vascular

tone and permeability (Kunder et al., 2011). Furthermore, tryptase and chymase, by promoting synthesis of MMPs may support tissue remodelling and ECM structure modification at time of menses (Pejler et al., 2007). Moreover, in contrast with other studies (Sivridis et al., 2001), the MCs detected in our study appeared to be phase-specifically activated in the myometrial layer the during secretory phase and at menses, indicating that MC mediators may play a role in arteriole sprouting during the secretory phase and muscle contraction during menses.

Due to the new data consistent with MC activation in particular phases of the cycle, the influence of sex steroid hormone on MC behaviour was also investigated. Evidence of the potential impact of steroid hormones on MC behaviour prior to the current study was reported by Drudy et al. (1991b). They used electron microscopy to demonstrate that MC numbers appeared to be significantly lower in uterine biopsies from postmenopausal women in whom sex hormones levels are low (Stone et al., 1975). Moreover, in that study an insufficiency in granule content in the MC cytoplasm was shown, and no activation (by evaluation of extracellular staining) was detected in any of the uterine compartments including the myometrium, suggesting that high levels of oestrogens and progesterone are required for the physiological activation/degranulation of MCs.

I believe this is the first study to carefully explore the capability of uterine MCs to respond to hormones, by directly investigating the MC immunoexpression of sex steroid receptors. Uterine MCs appeared to be $ER\alpha^{neg}$, $ER\beta^{pos}$ and PR^{neg} independent of the uterine compartment or phase of the menstrual cycle (Summary table in chapter 3). This result was in contrast to previous reports (Pang et al., 1995, Zhao et al., 2001, Nicovani and Rudolph, 2002), which demonstrated ERα and PR immunopositivity of human MCs in non-reproductive tissues including bladder and upper airways. Moreover, it is difficult to interpret the past results due to the employment of immunohistochemistry antibodies that are non-specific to the different isoforms of oestrogen receptors. Zhao et al. were the first authors to investigate expression of PR in human MCs but unfortunately the staining they performed on nasal polyps is questionable due to the poor quality of the published images.

The current findings suggest that high levels of progesterone are involved in triggering the degranulation of uterine MCs, and since MCs do not express PR, it is

believed that there is an indirect action of sex steroid hormones on MC phenotype. For example, from day 20, the stroma of the endometrium appears oedematous, in response to PGE2 and F2 secreted by endometrial stromal cells, and this process is known to be regulated by high levels of progesterone (Baird et al., 1996a). Furthermore, PGE_2 stimulates capillary permeability either directly or by means of increased histamine release which enhances the appearance of oedema in the stroma (Bergeron, 2000).

Interestingly, the activation of uterine MCs appears to be coincident with early stages of the secretory phase (early and mid). This specific temporal degranulation may be involved in the recruitment of other immune cells through secretions of chemoattractant factors such as TNFα and/or IL-8 (Moon et al., 2010), which occurs during the late secretory phase and after progesterone withdrawal (Maybin and Critchley, 2011).

6.3 Potential role for mast cells in endometriosis

In chapter 4, the potential role of MCs in endometriosis was investigated by analysing their phenotype in the three tissues that are predominantly altered in the condition: eutopic endometrium (functional layer), ectopic endometrium (endometriotic lesions) and the pelvic peritoneal wall. These studies revealed an abnormal MC phenotype in every tissue explored with detection of both MC_{TC} and MC_C in the eutopic endometrium and retention of this phenotype in the lesions. Investigation of MCs resident in the peritoneal wall was accomplished for the first time during this study. Notably peritoneal MCs had a MC_C phenotype with an intensely activated profile during normal conditions (no pain – no endometriosis), whereas in the peritoneum from women affected by chronic pelvic pain their phenotype was MC_{TC}, MC_T or MC_C and extracellular protease staining was not detected (no activation).

No previous studies have explored the phenotype of MCs in the eutopic endometrium of women with CPP or endometriosis. As detailed in chapter 3 and in previous studies, MCs resident in the functional layer of the normal endometrium were quite rare, and not detectable in every sample. Furthermore, in the functional endometrial layer of the control women MCs appeared to show a specific protease phenotype, MC_T, and no other phenotype was detected. These features were confirmed

in the biopsies from control patients in our endometriosis study (no pain – no endometriosis), however in two patients the eutopic endometrium was found to contain MC_{TC}. Notably this MC phenotype found in endometrial samples from the CPP and endometriosis patients independently of the phase of the menstrual cycle. The unexpected finding in the two "controls" may be explained by the finding that both women had previously undiagnosed endometriosis lesions and are should therefore be reclassified as "asymptomatic" endometriosis. A growing body of evidence indicates that the primary defect in endometriosis may be an alteration within the eutopic endometrium, and these findings support the concept of an environmental modification in the functional endometrium in endometriosis patients, which is having an impact on MC phenotype. It is notable that recent studies have demonstrated that the eutopic endometrium also presents with other immune cell abnormalities with macrophages reported to be higher in number and the ratio of M1/M2 subtype being increased suggestive of a higher percentage of "proinflammatory" cells (Braun et al., 2002, Berbic et al., 2009, Takebayashi et al., 2015). The MC phenotype "switch" is also supported by the evidence of an altered inflammatory protein production and gene expression in the endometrial microenvironment of women with endometriosis (Ulukus et al., 2006). For example, it has been demonstrated that the immunoexpression of IL-8 and its receptors was higher in endometrial biopsies from women with endometriosis than controls (Arici, 2002, Li et al., 2012) and IL-8 has been shown to exert a potent chemoattraction for mature MCs and other immune cells (Nilsson et al., 1999). We might also speculate that more hematopoietic progenitor cells might be recruited in the eutopic endometrium due to the increase of expression of CXCR2 (Ulukus et al., 2004), which is one of the key regulators of $CD34^+$ cell influx (Hallgren et al., 2007). To support this theory, it is notable that Franco-Murillo et al. (2015) have reported a significant increase in SCF in the functional endometrium from women with endometriosis, consistent with the presence of an endometrial environment predisposed to support MC proliferation and survival.

The complete phenotype of MCs in endometriotic lesions was not identified until the current study as although high numbers were detected in previous studies (Matsuzaki et al., 1998a, Sugamata et al., 2005), they did not explore the expression pattern of chymase. Remarkably, MCs identified in the endometriotic lesions strongly

expressed the MC_{TC} and MC_{C} phenotype, independent of the phase of the cycle or the grade of the disease, a finding that was in accordance with their phenotype in eutopic endometrial biopsies and would thus support the "retrograde menstruation" aetiology of endometriosis. The preliminary evidence provided in this study suggests MC number is increased in the lesions compared to the eutopic endometrium and appears consistent with sloughed endometrium from women with endometriosis providing a pro-differentiation and pro-survival environment for MCs. Additionally, MC survival/differentiation may also be augmented within the peritoneal microenvironment as peritoneal fluid has been demonstrated to contain elevated concentrations of SCF in women affected by endometriosis (Osuga et al., 2000).

The presence of MCs in the human peritoneum has been documented in nephrology reports describing the use of peritoneal dialysis and the associated development of fibrosis (Zareie et al., 2001). To date no publications have appeared describing MCs in healthy female peritonea or in cases of chronic pelvic pain and/or endometriosis. The exploration of MC phenotype in this study has brought new evidence that the pelvic peritoneal wall appeared to be altered in women affected by chronic pain and/or endometriosis compared to controls (no pain). Surprisingly abundant evidence of chymase in the samples from control women was almost absent in both groups of patients. Information presented in peritoneal dialysis studies (Jimenez-Heffernan et al., 2006, Stavenuiter et al., 2011, Lima et al., 2013), demonstrated that increased angiogenesis and fibrosis, both processes often reported in endometriosis, might result in peritoneal membrane damage. There are reports of an altered mesothelium permeability and increased osmotic pressure in the peritoneal cavity in women affected by endometriosis, as a result of higher levels of peritoneal fluid (PF; independently from the phase of the cycle) and an increased PF protein content (Syrop and Halme, 1987, Hurst and Rock, 1991, Oral et al., 1996b). Therefore, it is possible that an alteration induced by lesion formation (supported by angiogenesis) and an extensive inflammatory response may contribute to peritoneal damage and altered MC number and phenotype and this merits further investigation.

The pain associated with the growth of ectopic lesions in women with endometriosis is common and may be severely disabling (Triolo et al., 2013). Chronic

pelvic pain (CPP) is often resistant to standard analgesic treatments, probably because they do not directly address specific mediators that are involved in the endometriosis-associated pain. This is the first study to examine expression of the PAR-2 receptor in the pelvic cavity of patients suffering with CPP and/or endometriosis. PAR-2 is thought to play a critical role in inflammatory and visceral pain; it can be cleaved and consequently activated by MC tryptase and other proteases (Ossovskaya and Bunnett, 2004). Its activation controls neurogenic inflammation, pain and neuronal excitability (Amadesi et al., 2006). In this study, the immunoexpression of PAR-2 was extremely high in patients affected by CPP and/or endometriosis compared to no pain controls (with or without asymptomatic endometriosis) and this was independent of the stage of the disease. This novel finding extends and complements the nociceptive changes commonly detected in the peritoneum and lesions of women with endometriosis. For example, studies on the TRPV1 and TRPA1, ion channels expressed on neuronal central and peripheral projections (Bautista et al., 2006, Sipe et al., 2008) are both highly expressed in CPP/endometriosis patients compared to no pain controls (Poli-Neto et al., 2009, Rocha et al., 2011, Greaves et al., 2014b). Furthermore, it has been demonstrated that there is an interaction between PAR-2 and TRPV1 or TRPA1 in the establishment of inflammatory pain. For example, PAR-2 activation may sensitise neurons via TRPV1 stimulation and induce hyperalgesia (Amadesi et al., 2004, Terada et al., 2013). Thus this overexpression of PAR-2 and TRPV1 may be the cause of the generalized hyperalgesia reported by He et al. (2010) in women with endometriosis, which showed a much lower pain threshold compared to controls. The increased sensitivity to pain in CPP and endometriosis patients may also explain the results on MC counts and quantification of MC proteases in the peritoneal fluid of these women if similar numbers of MCs and low concentrations of MC neurogenic mediators, tryptase and histamine, can still trigger nociception due to increased sensitivity.

6.4 Current tools for investigation into mast cell phenotype and activation

Studies that set out to explore MC "behaviour" in using current *in vitro* and *in vivo* models have been described in chapter 5. Data gathered from *in vitro* differentiation of peripheral blood derived MCs were consistent with the *in situ* results, showing a full mature inflammatory MC phenotype at the end of the culture, with

expression of ERβ and GR. This has been the first study to interrogate the expression of steroid receptors during MC development from their progenitors to mature state *in vitro*. The differentiation method employed during the current study has provided evidence of the potential influence of oestrogens on different stages of $CD34^+$/MCs development, which is important for our understanding of $CD34^+$ recruitment and MCs maturation within the human uterus. Whilst the results obtained with peripheral blood derived MCs appeared to perfectly mirror the MC mature phenotype reported in the endometrium, the protocol followed (Kirshenbaum and Metcalfe, 2006) several limitations. It was extremely complex and time consuming and incurred a high expense considering the low MC numbers generated after an 8-week culture period. Additionally, only 5 out of the 20 cultures survived the entire culture period and hence this was not perceived as a viable and reliable method.

To overcome the limitations of the peripheral blood MC cultures, the leukaemia derived HMC-1 cell line, a well-established *in vitro* model (1988) was used. The phenotypical investigation conducted on HMC-1 cells during the current study, confirmed Nilsson et al.'s report (1994a), with a battery of molecular techniques showing that HMC-1 cells have an immature MC phenotype with low expression of proteases, tryptase and chymase consistent with the origins of the original cells. Surprisingly, the HMC-1 cells investigated using our standard in house culture systems (both with phenol-red and phenol-free media) revealed an $ER\alpha^{neg}$ and $ER\beta^{pos}$ phenotype which appeared in agreement with data from the studies in tissue samples and peripheral blood derived MCs although it appeared to contrast to previous reports using cells obtained from the same source. For example, HMC-1 cells have been extensively reported to express ERα, but the methods employed appeared to be non-specific. For example, by "blasting" the published primer sequences using the Basic Local Alignment Search Tool of the US National Library of Medicine (NIH), it appears that although some match the *ESR1* (gene encoding ERα) others correspond to *ESR2* instead (ERβ) (Ellem et al., 2014a). Furthermore, some of the studies showing immunopositivity for ERs on HMC-1 cells have employed antibodies that cannot distinguish between the two isoforms of oestrogen receptor (Pang et al., 1995, Letourneau et al., 1996). The HMC-1 cell line has two variants with a distinct set of

mutations of the c-kit receptor, thus HMC-1.1 and HMC-1.2, have one point mutation (V560G) or two point mutations (V560G and D816V) respectively (Sundstrom et al., 2003). As Dr Butterfield had generously gifted both of the two variants of HMC-1 cells, the phenotype of both was examined and there was no difference in the expression of ERs, with both variants $ER\alpha^{neg}$ and $ER\beta^{pos}$. In most of our studies HMC-1.1 were used due to their higher mRNA concentration of chymase compared to HMC-1.2. Interestingly, the results obtained investigating degranulation induced by oestradiol confirmed previous studies (Zaitsu et al., 2007), which showed HMC-1 cells did not respond to physiological E_2 concentrations of 10^{7}M and 10^{-8}M. The contradictions between the results in different studies may be explained by the culture conditions used or the passage number of the cells which are likely to vary between laboratories and/or operator. HMC-1 cells recapitulated the pattern of steroid receptor expression documented in the uterine MCs, and their proliferation rate is extremely high compared to primary cells however, their immature inflammatory state (low tryptase and chymase) limits their usefulness as a model for tissue resident MCs.

In chapter 5, the presence of MCs in the mouse uterus was explored both during the normal oestrus cycle and using an in house model of menstruation in which tissue breakdown, hypoxia, angiogenesis and remodelling have been documented, recapitulating key features of menses in women (Cousins et al., 2014). In the mouse uterus MCs were not detected in the endometrial compartment a result in contrast to that in women. The current study was conducted on Carnoy's fixed tissues as preliminary investigations conducted during optimisation of techniques showed that this fixative allowed for detection of both MC populations (mucosal and connective subtypes). Surprisingly the results obtained appeared to be consistent with other studies, which employed paraformaldehyde, that excludes "formalin sensitive" mucosal MCs (Woidacki et al., 2013b), suggesting that in mice uterine MC populations belong to only one of the murine MC subtypes, the connective type. This conclusion is further supported by a recent study, which used double-transgenic C57BL/6J Mcpt5-Cre ROSA26-EYFP mice, with high expression of enhanced yellow fluorescent protein in MC protease 5 (Cma1 (Mcpt5))-expressing cells in the uterus (Schmerse et al., 2014). Mcpt5 (or mMCP-5) is expressed by the connective MC subtype and mucosal MCs contain mMCP-1 and mMCP-2 chymases. Unfortunately,

these two different MC types don't share any of the MC specific proteases and therefore genetically modified mice do not fully recapitulate the phenotypes in women.

Despite our belief that the results using human uterine tissues suggest MCs may play a role in menstruation in our mouse model MCs did not appear to be involved in endometrial breakdown or tissue regeneration. However, in the mice MCs were consistently located near the muscle fibres in the myometrial layer, suggesting instead a role for MCs in myometrial hyperactivity raising the possibility that MCs may be implicated in primary dysmenorrhea, characterized by intense and acute abdominal pain during the first days of menstrual flow that affects up to 40-50% of women (Bulletti et al., 2000). A recent study by Yang et al. (2015) has recapitulated *in vivo* the clinical features of dysmenorrhea in a mouse model but did not investigate the role of MCs in the painful contractions. It would be interesting to study MC number in the myometrium in the animals of that model to explore whether compounds blocking activation of MCs can have an impact on behavioural tests.

To my knowledge, this was the first study that has demonstrated the presence of MCs in a mouse model of endometriosis (Greaves et al., 2014a). Similar to the human results in chapter 4, MCs were detected in the endometriotic "lesions" although in the mice MCs were absent in the "donor" material (endometrial tissue). Based on the current literature and the principle that MCs differentiate *in situ* from $CD34^+$ progenitors, I speculate that there might be two possible origins of the lesion-associated MCs: an endometrial or peritoneal origin of a $CD34^+$ cell pool which differentiated at the site of the lesions. Since $CD34^+$ cells are reported in the stromal compartment of the basal endometrium (Cho et al., 2004), it is feasible that cells resident in the basal endometrium and inner myometrium may become constituents of the lesions as collection of "donor" endometrial material involved scraping the endometrium from the myometrium with a scalpel. Alternatively, an interesting study on the "intraperitoneal foreign body reaction", a common reaction of the body against implanted materials like medical devices, reported the influx of $CD34^+$/c-kit^+ hematopoietic cells around the implanted devices (Vranken et al., 2008). Although the endometriosis tissue is not a "device", it is possible the reaction in the peritoneum is similar with recruitment of immune cells and ECM deposition. Further investigations

on $CD34^+$/MC origin need to be conducted but the mouse model offers one platform for studying MCs in endometriosis.

6.5 Study limitations

Some limitations of the study must to be highlighted for better address the future directions of project. Firstly, the use of human samples meant there was a relatively small sample size and high patient variability. Sample size was further reduced over the time of the project due to strict criteria for patient inclusion in an attempt to limit factors that would introduce more variability, as detailed in the methods sections of chapter 3 and 4. Another limitation encountered in chapter 3 was access to full thickness uterine sample biopsies. Total abdomen hysterectomy is a gynaecological surgical practice that is becoming less frequent and often replaced by medical treatment. A similar problem was encountered during the investigation of MCs in endometriosis, where it was challenging to collect 'no pain and no endometriosis' control samples. Laparoscopic sterilisation is a rare procedure in developed countries, where other methods of birth controls are available. Therefore, the sample size could not be easily augmented since new prospective tissue collection has been sporadic during the time of the study.

6.6 Future directions

The current study has brought together evidence that oestrogens may have an impact on the recruitment of progenitors and their *in situ* differentiation within the different uterine tissue layers. Based on the preliminary data in peripheral blood derived MCs it appears ERα may be activated during $CD34^+$ recruitment and during the first phases of MC differentiation, but binding to ERβ may be responsible for exerting oestrogen-dependent effects on mature MCs behaviour. Interestingly, the time-dependent expression pattern of oestrogen receptors in the cultures suggests that further investigations on full thickness sections of uterine tissue should evaluate the phenotype of MCs in the region, where MC progenitors and mature MCs are most abundant – the myometrial-endometrial junction and examine the $CD34^+$ during the different phases of the cycle. An exploration of the immunoexpression of ERα and ERβ on uterine $CD34^+$ cells could reveal whether the environment specific phenotype

is also true of progenitors or involves initial expression of ERα before a switch to ERβ during differentiation.

It is important to note that standard "flat" 2D culture systems may not be able to replicate the endometrial microenvironment (Ellem et al., 2014b). Thus it could be more appropriate to explore MC development in a 3D system to better mimic, in our case, the "uterine environment". In our laboratory, a 3D "endometrial" culture system has been well established with stromal and epithelial cells, using cell lines (SHT-290 and Ishikawa cells, Dr Simitsidellis personal communication), and this could be adapted by using a myometrial cells instead of epithelial cells. For example, uterine layers with a co-culture of myometrial cells and endometrial stromal cells might provide a platform for seeding $CD34^+$ (peripheral blood or cord blood derived) and exploring their differentiation and the impact of oestrogens and progestins. The 3D culture would be also useful to explore the abnormal MC phenotype by using cells from women with endometriosis, which are already available in our laboratory.

Studies carried out using tissues from women with CPP and/or endometriosis during chapter 4, have highlighted the potential that the impact of MCs on the pathophysiology of these conditions may be mediated via the PAR-2 receptor. These promising results need to be validated by using a larger number of samples particularly those from women who do not have CPP or endometriosis, as in the current study we found some of our small number of controls actually had asymptomatic endometriosis.

Notably, finding increased expression of PAR-2 in the human peritoneum and in the patient group has brought new evidence consistent with the neuronal sensitization in the peritoneum of women affected by CPP and/or endometriosis, and it may offer a valid therapeutic target in these women. It has also been reported that PAR-2 is upregulated in dorsal root ganglia in association with thermal hyperalgesia and cAMP-dependent neuronal hyper-excitability (Zhang et al., 2011, Huang et al., 2012), offering additional evidence of a link with pain. It is important that additional studies also investigate the cleaved form of the PAR-2, to inform a complete understanding of the activation status of the receptor. There are two antibodies available for PAR-2 (clones H99 and C17), other than the one used during the project

(clone SAM11, N-terminus specific), which recognise the extracellular loops or the intracellular domain of the receptor, both sites that remain unchanged after N-terminus cleavage by proteases. Its investigation could be further complemented using both of the mouse models employed during the investigations in chapter 5, which appeared to be promising platforms for further studies on implications of MCs in pain associated with common gynaecological conditions, such endometriosis or painful menstruation. As a first step it would be worth investigating whether the expression pattern of PAR-2 in human peritoneum and endometriotic lesions is recapitulated in the mouse model of endometriosis and if there is any expression in their dorsal root ganglia. Importantly experiments on PAR-2 antagonism and PAR-2 knockout animals have reported pain relief in mouse models of rheumatoid arthritis (Russell and McDougall, 2009) and irritable bowel syndrome (Cenac et al., 2007).

Unfortunately, the use of mouse models for MC studies has one major limitation; there are no antibodies currently available that recognise the MC specific proteases in this species. Therefore, the "old" method of toluidine blue staining is routinely employed for identification of murine MCs and was used in the current study. A significant step forward in the MC field was made by Schmerse et al. (2014) with the development of the C57BL/6J-Mcpt5-Cre ROSA26-EYFP mouse which labels connective tissue type MCs. As the evaluation conducted in the current project showed MCs resident in the uterus are of this subtype, the "Mcpt5" mouse may be a useful tool for exploring the role of MCs in both our menstruation and endometriosis models.

6.7 General conclusions

Mast cells are a component of the leukocyte population of the human uterus and it has been demonstrated that MCs may play an important role in both physiology and pathology of the endometrium. Mast cell activation/degranulation is menstrual cycle stage dependent, and their environment-specific phenotype has suggested the oestrogens influence in progenitor/MC development, via different ER receptors. This study has provided an insight into the potential that MCs may play a role in formation of the endometriotic lesion and the establishment of chronic pain and hyperalgesia. The preliminary results from mouse models have offered a valid platform for further

studies on the implications of MCs in pain associated with endometriosis or menstruation.

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Presentations relating to this thesis

Society for Reproductive Investigation - 62nd Annual meeting

March 2015, San Francisco, USA.

Poster: "Investigation of the steroid hormone receptors and activation profile of uterine mast cells" (Poster).

Society for Reproductive Investigation - 63rd Annual meeting

March 2016, Montreal, Canada.

Poster: "Mast cells and chymase: are they partners in crime in pelvic pain and endometriosis?"

Society for Reproductive Investigation – 64th Annual meeting

March 2017, Orlando, USA.

Poster: "Possible role for mast cells and PAR-2 receptor in the hyperalgesic state of women with endometriosis"

Training courses and awards

Society for Reproductive Investigation (SRI) – Poster Award $100 (March 2013).

"11th College of Medicine and Veterinary Medicine Microscopy course", at University of Edinburgh, October 2013.

Medical Research Council Centenary Award – Training Grant £1500 (October 2014) to attend the "Course in Flow Cytometry", University of York, January 2015.

Society for Reproduction and Fertility (SRF) – Travel Grant £375 (July 2015) to attend SRI 62nd Annual Meeting, San Francisco 2015.

British Society for Immunology (BSI) – Travel Grant £1000 (December 2015) to attend SRI 63rd Annual Meeting, Montreal 2016.

Society for Reproductive Investigation (SRI) – Poster Award $100 (March 2016).

University of Edinburgh, Deanery of Clinical Sciences Funding Challenge Award - £2490 (April 2016)